Search for nicotinic acetylcholine receptors on lymphocytes

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SEARCH FOR NICOTINIC ACETYLCHOLINE RECEPTORS ON LYMPHOCYTES

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For my Dad, whom I love and miss.

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ABBREVIATIONS

ACh	Acetylcholine
AChE	Acetylcholinesterase
AChR	Acetycholine receptor
cAMP	cyclic 3', 5'- adenosine monophosphate
α-BGT	α-Bungarotoxin
BSA	Bovine serum albumin
BSS	Balanced Salt Solution
BZQ	Benzoquinonium chloride
C2	Complement factor 2
C3	Complement factor 3
cDNA	Complementary deoxyribo-nucleic acid
Con A	Concanavalin A
CNBr	Cyanogen bromide
CNS	Central Nervous System
DMPP	1-1-dimethyl-4-phenyl piperazium
DMSO	Dimethylsulphoxide
EAMG	Experimental autoimmune myasthenia gravis
EDTA	Ethylene diamine tetra-acetic acid
ELISA	Enzyme-linked immuno-sorbent assay
EJ	extrajunctional
EPP	End-plate potential
FCA	Freund's complete adjuvant
FCS	Fetal calf serum
FIA	Freund's incomplete adjuvant
cGMP	cyclic 3', 5'- guanosine mono phosphate
HAT	Hypoxanthine, amino-pterin thymidine
HBSS	Hanks Balanced Salt Solution
IgG	Immunoglobulin G
IgM	Immunoglobulin M
JR	Junctional receptor
Mab	Monoclonal antibody
mAChR	Muscarinic acetyl-choline receptor
MEPP	Miniature end-plate potential
MG	Myasthenia Gravis
MHC	Major histo-compatability complex
MIR	Main immuno-dominant region
mRNA	Messenger ribonucleic acid
nAChR	nicotinic Acetyl-choline receptor
NMJ	Neuro-muscular junction
NMS	Normal mouse serum
NP-40	Nonidet-P40

NRS	Normal rabbit serum
O.D.	Optical density
PBL	Peripheral blood mono-nuclear leucocytes
PBS	Phosphate buffered saline
PEG	polyethylene glycol
PEI	polyethyleneimine
pI	Isoelectric point
PMSF	Phenylmethyl-sulphonyl fluoride
RIA	Radioimmunoassay
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SRBC	Sheep red blood cells
TBA	Toxin binding assay
TCA	Trichloroacetic acid
TMB	3, 3', 5, 5'-tetra-methylbenzidine
T_H	T helper cell
T_S	T suppressor cell

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SUMMARY

Nicotinic acetycholine receptor (nAChR) was purified from *Torpedo* electric organ and fetal calf muscle and used to raise polyclonal and monoclonal antibodies, with a view to their use as probes for nAChRs on lymphocytes.

Rabbits immunised with fetal calf nAChR developed symptoms typical of Experimental Autoimmune Myasthenia Gravis (EAMG) and yielded polyclonal antisera (F1 and F2) which were characterised in terms of their specificities towards nAChRs from different sources.

Enzyme-linked immunosorbent assays (ELISAs) were developed to screen for and assay monoclonal antibodies raised against both *Torpedo* and fetal calf nAChR. Five stable hybridoma lines were established, following immunisation of mice with *Torpedo* nAChR; the corresponding monoclonal antibodies (B11, C7, C11, E8 and E11) were of IgG subclass and showed single site, high-affinity binding to *Torpedo* nAChR. They all interacted with fetal calf and human muscle nAChR while monoclonal antibodies B11, C11 and E8 also recognised nAChR expressed on cultured rat myotubes. The specificities of monoclonal antibodies towards nAChR subunits were also determined. Immunisation of mice with fetal calf nAChR yielded anti-nAChR secreting hybridomas but no stable cell lines were established.

The polyclonal antiserum F2 and monoclonal antibodies, B11, C7 and E11, showed specific interreactions with human peripheral

blood lymphocytes (PBLs), as assessed by ELISA and Solid Phase radioimmunoassay.

The presence of AChRs on lymphocytes was also sought by using radiolabelled ligands. [^{125}I]-α-bungarotoxin, used as a probe for nAChR, was found to bind specifically to mouse thymocytes in only one of many attempts; no specific sites were detected on human PBLs. [^{3}H]-QNB, a probe for muscarinic AChRs, was found to bind specifically to mouse thymocytes and to human PBLs and binding characterisitcs were determined. [^{3}H]-Nicotine apparently bound to mouse thymocytes and to human PBLs. Binding to human PBLs was consistent but anomalous in its response to classical nicotine ligands. Further studies showed that [^{3}H](-)-nicotine did not bind to isolated PBL membranes and lysis experiments finally confirmed that the 'binding' of [^{3}H](-)-nicotine to PBLs could be explained in terms of uptake of radiolabel.

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INTRODUCTION

1. General Introduction

It is now well established that neuromuscular transmission at the motor endplate of vertebrate muscle is mediated through the interaction of the neurotransmitter acetylcholine (ACh) with the nicotinic acetylcholine receptor (nAChR). This receptor has been the subject of intensive research activity over the past 10 years. The receptor protein has been particularly well characterised to the point where the genes coding for its constituent peptides have been sequenced.

This progress can be attributed largely to the availability of a rich source of nAChR from the electric organs of electric fish, which has provided a stepping stone for the study of nAChRs of vertebrate muscle. The involvement of the nAChR as the autoantigen in the autoimmune disease, myasthenia gravis (MG) has provided further impetus to the study of this receptor. However, in contrast to the wealth of information about the purified nAChR, there has been relatively little progress in the understanding of the initiation of the autoimmune response in the disease.

Antibodies raised against the nAChR, in particular monoclonal antibodies (Mabs), have been invaluable in dissecting and analysing important structural and functional features of the receptor. They are now finding a use in the identification and

purification of nAChRs from the central nervous system (CNS) and have helped to demonstrate the presence of multiple types of nAChR which differ from muscle AChR in structure and pharmacology. There have also been suggestions that nicotinic receptors occur on cells of the immune system and the generation of antibody probes for such receptors form the basis of this thesis. It is therefore appropriate to review the current status of the nAChR, its involvement in MG and the production and use of Mab probes in the study of the AChR.

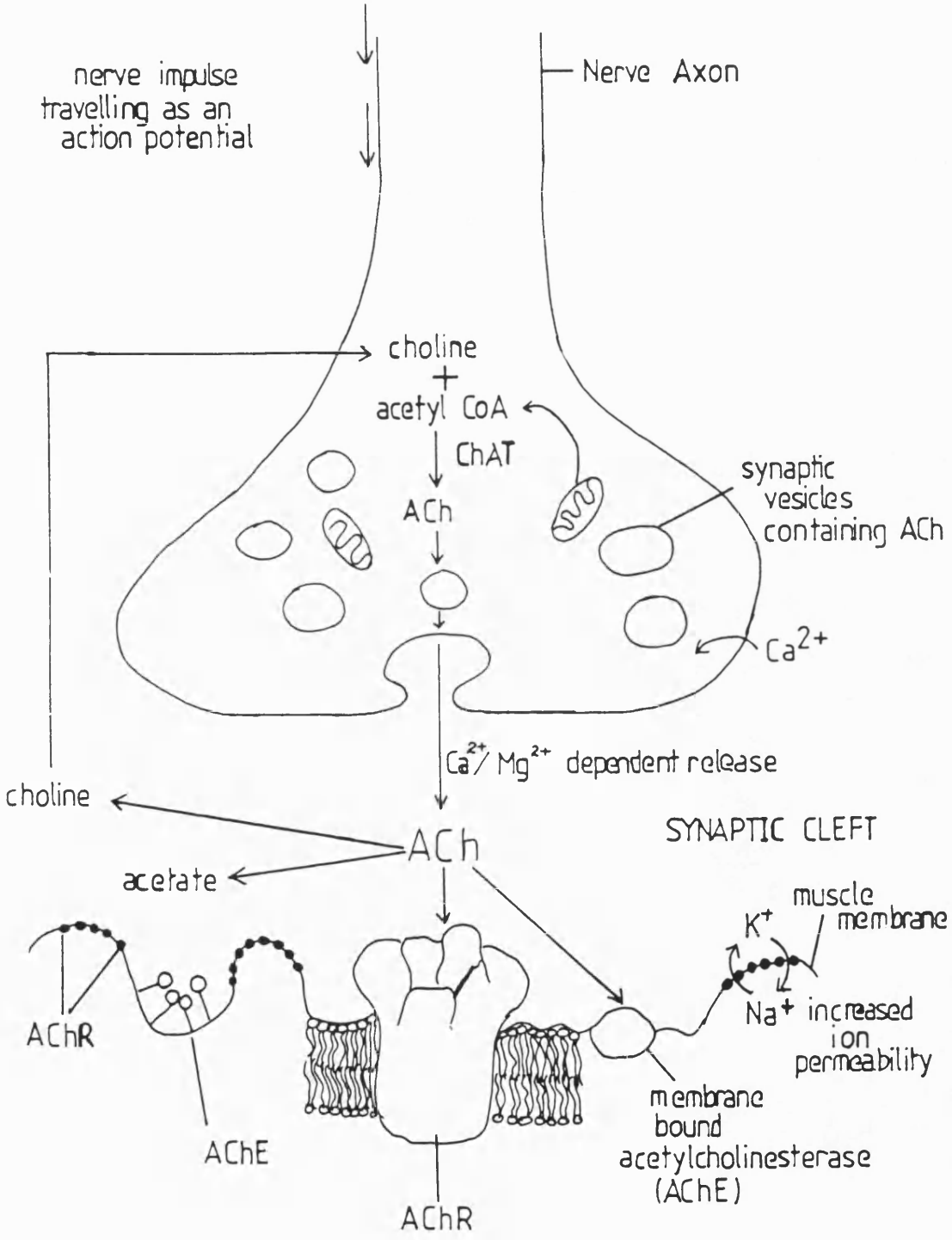
2. The nAChR

2.1 Neuromuscular transmission

The role of neuromuscular transmission is to transfer the propagated nerve impulse from the motor nerve ending to the muscle fibre, resulting ultimately in muscle contraction. It is the function of the AChR to translate a chemical signal into an electrical response. In the majority of vertebrate muscles, each muscle fibre is innervated by one nerve, and the area of contact between the two is termed the neuromuscular junction (NMJ). Figure 1 (page 3) gives a schematic representation of the NMJ.

Neuromuscular transmission is initiated when an action potential invades the nerve terminal. The resultant inward flow of Ca^{2+} ions, through voltage dependent Ca^{2+} channels, triggers the release of ACh, which diffuses across the synaptic cleft. The

Figure 1 Schematic organisation of the neuromuscular junction



binding of ACh to the nAChR in the post-synaptic membrane results in a conformational change which triggers the transient opening (1-2msec) of a cation-selective ion channel. This mechanism is thought to occur by allosteric transitions (see Introduction, page 6). The channel allows Na^+ and K^+ ions to flow down their respective electrochemical gradients. More Na^+ move in than K^+ ions move out, resulting in a net influx of positive charge. This local depolarisation produces a miniature end-plate potential or MEPP. MEPPs reflect the random release of one quanta of transmitter or the entire content of one vesicle.

If the nerve impulse is strong enough many quanta (100-200) are released and the MEPPs summate to produce an end plate potential (EPP) significantly shifting the membrane potential at and around the synapse. As the membrane potential approaches the threshold value (-15mV), an action potential is generated and spreads over the entire muscle causing the muscle to contract. The action of ACh is terminated by its dissociation from the AChR, and enzymic hydrolysis by acetylcholinesterase (AChE). Membrane potentials are re-established by membrane-bound Na^+ / K^+ ATPases.

2.2 Pharmacology

Cholinergic receptors, that is receptors which interact specifically with ACh, were divided at an early date by Dale (1914) into two different types, the nAChR and muscarinic AChR

(mAChR). Nicotine is ineffective at the mAChR and conversely muscarine has no effect at a nicotinic synapse. The response of mAChR to muscarine is slow in onset and prolonged ($\sim$100ms) and can be blocked by atropine. In contrast, the agonistic activity of nicotine at the nAChR is characterised by a fast response (1-2ms) and its effects can be blocked by d-tubocurarine. nAChR and mAChR are widely distributed throughout the nervous system. In general, muscarinic synapses are found in smooth and cardiac muscle and brain, whereas nAChR are located at autonomic ganglia and at skeletal NMJs.

In general, cholinergic ligands which act at the nAChR can be broadly classified into two categories :-

Agonists - which bind at the agonist binding site and activate the receptor.

Antagonists - agents which prevent the action of agonists.

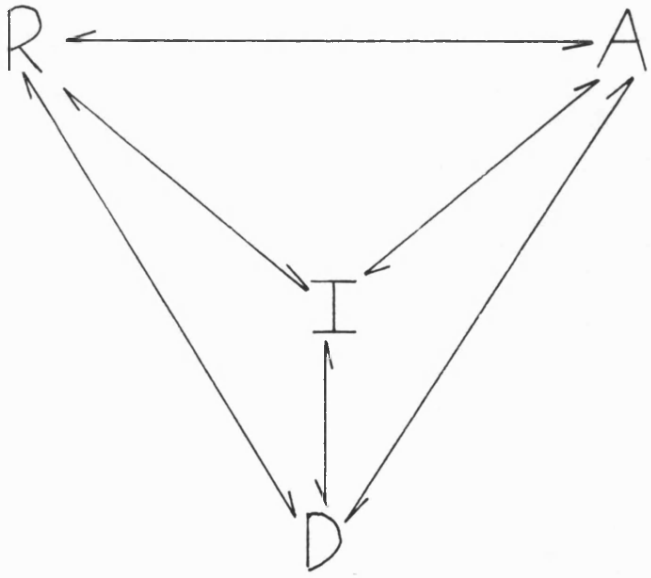
2.2.1 Agonists

These include nicotine, ACh and carbamylcholine. The nAChR is stereoselective with respect to nicotine, the naturaly occurring isomer (-) - nicotine being more potent than (+) - nicotine.

Prolonged exposure of AChR to agonists promotes desensitisation, or the conversion of the receptor into an inactive state where the ion channel is closed. This has led to complications in the classification of nicotinic agonists as, under these conditions,

they have antagonistic characteristics. Furthermore, drugs can vary in their effectiveness in promoting desensitised blockade.

Changeux in 1981 proposed a model which describes receptor function in terms of allosteric transitions between various states induced by cholinergic ligands. This model is shown below and illustrates the process of desensitisation.



The binding of agonist, to the receptor in its resting (R) state triggers the opening of the ion channel (active A state). Prolonged exposure of AChR to agonists provokes a two step desensitation process to the I (intermediate or rapidly desensitised state, $\sim$ 100ms) and to the D (slowly desensitised state, $\sim$ sec-min). All the states are discrete and interconvertible with some of the states being present before ligand binding. The affinity of the receptor for agonist increases from state R to D via the states A and I. The forward rate of desensitisation is dependent upon the agonist and its concentration whereas the rate of recovery from desensitisation is independent of the agonist.

2.2.2 Antagonists

Nicotinic antagonists can be subdivided into two major classes:-

a) Competitive inhibitors - which simply compete for the agonist binding site. At the vertebrate NMJ these agents include d-tubocurarine, di-hydro-β-erythroidine (DHβE) and the α-neurotoxins, the most potent of which is α-Bungarotoxin (α-BGT).

b) Non-competitive inhibitors which prevent activation of the nAChR by binding to sites distinct from the ACh binding site include local anaesthetics, the hallucinogen phenyclidine, non-ionic detergents and the toxin histrionicotoxin isolated from the Columbian tree frog Denrobates histrionicus. There are different affinity sites for non-competitive inhibitors and they inhibit

receptor function by one of the following mechanisms :- stabilisation of the resting state, blocking the open ion channel or promoting desensitisation via allosteric interactions.

Finally, nicotinic receptors at the NMJ and at autonomic ganglia differ in their sensitivities to drugs and have been subdivided on the basis of their sensitivities to hexamethonium and decamethonium. Ganglionic receptors are sensitive to hexamethonium and are referred to as C6 receptors whereas receptors at the NMJ are sensitive to decamethonium and are referred to as C10 receptors. Other drugs have also been used to differentiate between the two groups, eg: 1,1-dimethyl-4-phenyl-piperazinium (DMPP) is a specific agonist for C6 receptors whilst α-BGT is only antagonistic at C10 receptors (see also Introduction, page 32).

Decamethonium which was originally used in the early classification of C10 receptors acts as a depolarising agent at the NMJ first stimulating the receptor (thereby acting as an agonist) but then causing desensitisation.

Major advances in the biochemical isolation of the nAChR lagged behind the pharmalogical characterisation of the receptor and only became feasible following the identification, by Lee and co-workers, of certain elapid snake neurotoxins (α-neurotoxins) as very specific, high-affinity probes for the ACh binding site (reviewed by Lee, 1979).

2.3 The α-neurotoxins

The α-neurotoxins are small basic polypeptides with (Mr$\sim$ 8000) and have been classified into two groups on the basis of their behaviour at the NMJ.

Type 1 - exert a reversible neuromuscular block and have an important application in affinity purification of AChR (eg: the α-toxin from Naja naja siamensis venom). Neurotoxins of this class have 60-62 amino acid residues.

Type 11 - neurotoxins have 71-74 amino acids and bind almost irreversibly to the AChR. These toxins, particularly α-BGT from the krait Bungarus multicinctus, can be radiolabelled to high specific activity and have been used extensively to detect and quantitate AChR in biochemical analyses. The nAChR content of a tissue source is usually quantified in terms of moles of α-BGT binding sites per gram of protein.

Biochemical studies of the nAChR benefited further from the recognition that the electric organs of electric eels (Electrophorus electricus) and electric fish (Torpedo genus) are extremely rich sources of AChR. The electric organ is phylogenetically evolved from striated muscle and the nAChR from these sources is generally assumed to have all the important features of vertebrate muscle nAChR. However, nAChRs can be obtained in 1000-fold larger quantities from electric organ tissue (~100mg/kg) than from innervated muscle (<0.5mg/kg) and accordingly the receptor from electric fish has been extensively

used for biochemical characterisation (for reviews see Anholt et al., 1984, Stroud and Finer-Moore, 1985, Hucho, 1986, McCarthy et al., 1986). Extensive reviews exist and only a concise account of the present understanding of the nAChR will be given here.

2.4 Purification of nAChR

Purification of nAChR relies on the extraction of the receptor protein from its membrane environment using detergent (eg: Triton X-100, deoxycholate). Specific isolation of the receptor has relied on affinity chromatography using Type 1α-neurotoxins (Introduction, page 9), agonists, antagonists and AChR-specific Mabs (Introduction, page 22). In some cases further purification has been carried out by using immobilised lectins (see Anholt et al., 1984 for review).

In the isolation of nAChR from vertebrate muscle, advantage has been taken of denervation (see Introduction, page 14) which results in a large increase in AChR (reviewed by Dolly, 1979), making it a richer source of receptor.

Purification procedures employ extensive use of anti-proteolytic agents because of the susceptibility of the receptor protein to proteolysis during purification (Lindstrom et al., 1980; Schorr et al., 1981; Einarson et al., 1982). The problems of receptor density and proteolysis become particularly important in the purification of AChR from other sources such as the central nervous system (CNS) and lymphocytes.

2.5 Characterisation of nAChR

The nAChR from Torpedo electric organ and vertebrate muscle is a glycoprotein composed of 4 different polypeptide chains assembled into a heterologous transmembrane pentamer ($\alpha 2, \beta, \gamma, \delta$). From sodium dodecylsulphate/polyacrylamide gel electrophoresis (SDS/PAGE) the fish receptor subunits have respective Mr values of 40K, 50K, 60K and 65K. Although controversy initially surrounded the subunit pattern of vertebrate muscle nAChR (reviewed by Conti-Tronconi and Raftery, 1982) evidence from gene cloning (see Introduction, page 17) confirmed the same subunit structure. However, the subunit Mr values of muscle nAChR are slightly different from those of their fish counterpart (for review of vertebrate muscle nAChR see Dolly and Barnard, 1984).

The AChR is a phosphoprotein (Vandlen et al., 1979) with the phosphate groups located on the cytoplasmic face of the receptor (Huganir et al., 1984, see also Table 1 page 12). The functional significance of phosphorylation is unknown although it may be involved in receptor desensitisation (see Introduction, page 5). The degree of phosphorylation of AChR changes during ontogenic development, adult receptor being more phosphorylated than its immature counterpart (Saitoh and Changeux, 1981). The interconversion is thought to occur by phosphorylation-dephosphorylation reactions catalysed by protein kinases and protein phosphatases (see Anholt et al., 1984). Pizzaghella et al., (1983a) have also demonstrated the presence

Table 1 Properties of purified AChR from Torpedo Electric Fish

Property		Reference
Structure		
Subunit composition : $\alpha\ \beta\ \gamma\ \delta$		
Mr values polypeptide chains (by SDS PAGE): (40,000), (50,000), (60,000), (65,000)		Conti-Tronconi and Raftery,1982
Mr values polypeptide chains (from CDNA sequences) (50,200), (53,700), (56,300), (57,600)		Numa et al., 1983
Physical Properties		
Sw_{20} value	9S MONOMER 13S DIMER	Reynolds and Karlin, 1978
Isoelectric point (α-BGT complex)	4.9	Raftery et al., 1972
Diameter of receptor complex by electron microscopy	8.5nm	Cartaud et al., 1978
Carbohydrate content		
75 residues/molecule Glucosamine, mannose, glucose, galactose		Vandlen et al., 1979
1 mole sialic acid per mole receptor		Bersinger et al., 1983
Phosphorylation state		
Contains 9 phosphoserines distributed 1,1,2,5 (α, β, γ, δ) respectively		Vandlen et al., 1979

of the phosphorylated protein, phosvitin, by Mab binding. Methylation of the nAChR has also been reported however its physiological role is not yet established (see Hucho, 1986).

Several non-receptor proteins, mainly of 43K, are associated with the AChR and these may play a role in receptor stability and synapse formation (see Anholt et al, 1984 for review). One 43K protein can be chemically cross-linked to the β-subunit of nAChR (Burden et al., 1983) and is co-extensively distributed with the receptor (Sealock et al., 1984).

The nAChR from Torpedo exists in monomeric and dimeric forms with sedimentation coefficients of 9S and 13S (Reynolds and Karlin, 1978). Monomers are linked covalently by disulphide bridge(s) between the δ units to form a dimer (Sobel et al., 1977; (see also Figure 3, page 24); the disulphide linkage may be extracellular (Dunn et al.,1986), with the dimeric form of the receptor representing the functional unit in vivo (Schindler et al., 1984). The dimeric form of nAChR has only been observed in Torpedo species. Table 1 (page12) gives a summary of the properties of purified Torpedo nAChR.

Vertebrate nAChRs have a sedimentation coefficient of 9S, with a smaller α-BGT binding species of 4-5S sometimes also observed (Lo et al., 1981; Sumikawa et al., 1982a). However, the characterisation of vertebrate nAChR is complicated by the presence of two distinct forms of receptor, junctional (J) and extrajunctional (EJ).

Fetal and neonatal muscle fibres express nAChR over their entire surface (Bevan and Steinbach, 1977). Innervation results in clustering of nAChR below the nerve terminal and decrease of receptors at extrajunctional sites. At the adult synapse therefore J receptors are highly concentrated in the post-synaptic muscle membrane (Fambrough, 1979; see also Figure 1, page 3). In contrast, receptors localised at sites outside the synapse, EJ receptors, are barely detectable in normal adult innervated muscle.

Denervation of muscle causes the proliferation of EJ receptors which become diffusely distributed over the muscle fibre surface. Hence, denervated muscle becomes a richer source of nAChR (see Harrison and Behan, 1986 for comparison of the [^{125}I]-α-BGT binding capacity of innervated and denervated muscle sources). EJ receptors differ, in several respects from J receptors (summarised in Table 2, page 16). The receptor types are indistinguishable by gel filtration and sucrose density gradient centrifugation (Brockes and Hall, 1975 ; Froehner et al., 1977; Nathanson and Hall, 1979) indicating that the differences are subtle. Different antigenic properties of J and EJ receptors have been reported (Weinberg and Hall, 1979). Post-translational modifications such as phosphorylation (Saitoh and Changeux, 1981; see also Introduction, page 12) may also contribute to the biochemical differences observed.

The mechanism underlying the difference in channel properties of the J and EJ receptors has recently been studied. Calf muscle AChR has been shown to contain an ε subunit which shows extensive homology with the γ subunit (Takai et al., 1985). The channel properties of expressed receptor are defined by the presence of either the ε subunit (adult channel characteristics) or γ subunit (embryonic channel characteristics) (Sakmann et al., 1985). Indeed results suggest that bovine embryonic nAChRs have an $\alpha 2, \beta, \gamma, \delta$ subunit composition whereas adult AChRs have an $\alpha 2, \beta, \gamma, \varepsilon$ subunit structure (Schuetze, 1986). These studies were made possible by the availability of cDNA probes from cloning.

2.6 Cloning the nAChR

The cDNAs coding for the α-chain in Torpedo marmorata, for the four subunits in Torpedo californica and calf skeletal muscle and the α and γ chains of human skeletal muscle, have all been cloned and sequenced (see Table 3, page 17 for references). By use of a calf γ-subunit cDNA probe, a fifth type of nAChR subunit (ε) has been cloned and sequenced from fetal calf muscle, (see Introduction, page 17).

Valuable information has been obtained from the cDNA sequences of the four subunits which have shown the subunits to be highly homologous (from 10-60% with an average of 40%) (Noda et al., 1983b) confirming data from amino-acid sequences (Raftery et al., 1980) and Mab studies (see Introduction, page 21). This supports the suggestion that the subunits have descended from a

Table 2 Biochemical and Biophysical Difference between Junctional and Extrajunctional Receptors

Property	Characteristic Details	Reference
Mobility in Membrane	J receptors - Immobile EJ receptors - Exhibit lateral-mobility	
Rates of Turnover in Membrane	J receptors - Half lifetime $\sim$ 5-9 days EJ receptors - Half lifetime $\sim$ 20 hours	Anholt et al., 1984 and references therein
Mean channel opening time	J receptors - $\sim$ 1ms EJ receptors - $\sim$ 2-3ms	
Differences in Isoelectric point	J receptors - pI toxin complex 5.1 EJ receptors - pI toxin complex 5.3	Brockes and Hall, 1975 (see also Turnbull et al., 1985)
Dissociation constant for d-Tubocurarine binding	J receptors - Kd 4.5 x 10^{-8} M EJ receptors - Kd 5.5 x 10^{-7} M	Brockes and Hall, 1975
Differences in glycosylation	J receptors are more glycosylated than EJ receptors	Turnbull et al., 1985

a) from rat diaphragm muscle
c) from human fetal and adult skeletal muscle

Table 3 Cloning of the nAChR genes

SUBUNIT CLONED	SPECIES OF SUBUNIT CLONED	REFERENCE
γ	T.californica	Ballivet et al.,1982 Claudio et al.,1983
α	T.marmorata	Sumikawa et al., 1982b Devillers-Thiery et al., 1983.
α	T.californica	Noda et al., 1982
β δ	T.californica	Noda et al., 1983a
α	Calf skeletal muscle	Noda et al., 1983c
β	Calf skeletal muscle	Tanabe et al., 1984
γ	Calf skeletal muscle	Takai et al., 1984
δ	Calf skeletal muscle	Kubo et al., 1985
ε	Calf skeletal muscle	Takai et al., 1985
α	Human skeletal muscle	Noda et al., 1983c
δ	Human skeletal muscle	Shibahara et al., 1985

Gene isolation depended upon a number of factors : the high abundance of mRNA from Torpedo coding for the subunits (Mendez et al., 1980), amino terminal protein sequence data (Devillers-Thiery et al., 1979; Raftery et al., 1980), subunit-specific antibodies to each of the four chains (Claudio and Raftery, 1977; Lindstrom et al., 1978), and the ability of the Xenopus oocyte to translate, process and assemble functional AChR when injected with electric organ mRNA (Sumikawa et al., 1981; Mishina et al., 1984; Sakmann et al., 1985).

The availability of cDNAs from Torpedo enabled the detection of homologous sequences in libraries of cDNA clones derived from RNA from vertebrate muscle using hybridisation techniques.

four-fold duplication of a single ancestral gene (Conti-Tronconi et al., 1985).

Analysis of the primary structures revealed 4 hydrophobic domains homologous in all polypeptide chains (α_2, β, γ, δ) of the nAChR and a fifth domain with alternating polar and hydrophobic amino acids. This latter domain is postulated to form an amphipathic helix with a hydrophilic core contributing to the ion channel formed in total by the homologous amphipathic helices (amino acid residues 425-451), from all 5 polypeptide chains. These features are the basis of the model proposed by Finer-Moore and Stroud (1984). Alternative structures have been proposed as a result of monoclonal antibody studies and are discussed later (Section 3).

2.7 Functional domains of the nAChR

Injection into Xenopus oocytes of mRNAs coding for the α, β, γ, δ subunits has confirmed that all five subunits contribute to the ion channel (Mishina et al., 1984). The β subunit may have a channel-gating function (Blatt et al., 1986). Injection of mRNA corresponding to cDNA specifically altered at predetermined sites, into ooctyes has demonstrated that the residues α192 and α193 are important at the agonist binding site and that the N-glycosylation site at asparagine residue α-141 is important for the assembly and function of the receptor (Mishina et al., 1985). However, the effects of amino acid substitution or deletion may be deleterious to the tertiary

structure of the receptor and hence the findings must be treated cautiously. These findings have, however, been substantiated by determining the reaction site of the affinity labels 4-(N-maleimido)-benzyltrimethyl ammonium (MBTA) (Karlin, 1969) and bromoacetylcholine (BAC) (see McCarthy et al., 1986) which bind covalently to the ACh binding site on the α-subunits after reduction of di-sulphide bonds. Under mild conditions of reduction, only one α-subunit is labelled per monomer (Weill et al., 1974; Damle et al., 1978; Damle and Karlin, 1978) but under harsher conditions both sites can be labelled (Wolosin et al, 1980; Walker et al., 1984) suggesting non-equivalent ACh binding sites (see Ratnam et al., 1986c).

The cysteine (cys) residues α192 and α193 are the target of MBTA (Kao et al., 1984; Kao and Karlin, 1986), and are disulphide cross-linked to each other. These residues are unique to the α-subunit in terms of sequence homology (Numa et al., 1983). The cys residues α128 and α142 are also disulphide cross-linked (Kao et al., 1984, Kao and Karlin , 1986). The presence of a double di-sulphide bond is unique to the α-subunit.

Synthetic peptides and antibodies specific to regions of the α-subunit of T.californica have implicated the amino acid residues 125-147, 173-204 and 185-196 (Wilson et al., 1985; Mulac-Jericevic and Atassi, 1986; Neumann et al., 1986) as the location of the α-BGT binding site on the α-subunit. Proteolysis studies of the α-subunit indicate that the

N-glycosylation site at asparagine residue α141 is near to but not part of the agonist binding site (Wilson et al., 1985; Oblas et al., 1986).

Despite the considerable homology between vertebrate muscle and electric fish nAChRs, evidence suggests that the ACh binding site of human muscle nAChR is structurally dissimilar from that of Torpedo electric organ, having a higher affinity for the affinity label BAC and an adjacent disulphide bond that is more readily accessible to reducing agents (Momoi and Lennon, 1986).

2.8 Shape of the nAChR

Estimates of the dimensions of the nAChR in the membrane have been obtained from X-ray scattering data and immuno-electron microscopy studies (reviewed by Hucho, 1986). The five membrane-spanning subunits appear to lie at pentagonally symmetrical positions arranged in a cylindrical rosette to form a central pore through which ions flow (Brisson and Unwin, 1985, Kubalek et al., 1987, see also Figure 3, page24). The overall length of the receptor from T. marmorata has been estimated to be 110Å, extending 15Å into the cytoplasm and 55Å extracellularly.

In summary, many techniques have proved useful in the study of nAChRs from fish and vertebrate muscle sources. Another technique, the production and use of anti-(nAChR) specific Mabs have also greatly advanced the characterisation of the nAChR.

3. The production and use of polyclonal and monoclonal antibodies in the study of nAChRs.

Many groups of workers have raised nAChR-specific Mabs (see Table 4, page22). Mabs have several advantages over polyclonal antisera, but both have proved useful in the study of the nAChR. Mabs are available in relatively endless supply and are monospecific although limitations follow from their advantages. For example, a Mab may be so specific that it does not bind to the part of the antigen of interest. Alternatively, the affinity of binding might be undesirable. For instance, high affinity Mabs are not suitable for immunoaffinity chromoatography because it is difficult to recover the bound antigen. The Mab produced may show undesired cross-reactivity with an irrelevant epitope or may not cross-react with a related antigen. These limitations, however, have been overcome by developing libraries of Mabs.

Before discussing the usefulness of Mabs as probes of the nAChR it is relevant here to summarise briefly Mab production and characterisation.

Mab production is based on the clonal selection hypothesis of Burnet (1959) which states that each B-lymphocyte may produce only one specific immunoglobulin which is genetically predetermined. Present day methods of Mab production have been modified from those originally described by Kohler and Milstein (1975) and are well documented in a review by Campbell (1984). The major practical steps involved in making Mabs are outlined

in Figure 2 (page23). For the production of Mabs several myeloma fusion partners are available (see Samoilovich et al., 1987). A common fusion partner used in the production of mouse/mouse hybridomas is the HAT sensitive non-immunoglobulin secreting cell line P3-X63-Ag8.653 (X63, Kearney et al., 1979).

Table 4: Monoclonal antibodies to the nAChR

a AChR species used as Immunogen	b Reference
Torpedo californica :	Gomez et al., 1979 Lennon and Lambert, 1980 Tzartos and Lindstrom, 1980 Mochley-Rosen & Fuchs, 1981
Torpedo californica : denatured AChR and purified subunits	Tzartos and Lindstrom, 1980 Froehner et al., 1983
Torpedo marmorata :	James et al., 1980 Watters and Maelicke, 1982 Whiting et al., 1985
Narcine brasiliensis :	Dwyer et al., 1981
Electrophorus electricus :	Tzartos et al., 1981
Bovine fetal calf	Tzartos and Lindstrom, 1981
Human skeletal muscle	Garabedian and Morel, 1983 Tzartos et al., 1983 Whiting et al., 1986c
Chick muscle	Mehraban et al., 1984

a unless other wise stated native AChR was used as immunogen for Mab production

b only the initial reference quoting the production of the relevant Mab is cited

Figure 2 The principal steps in making monoclonal antibodies

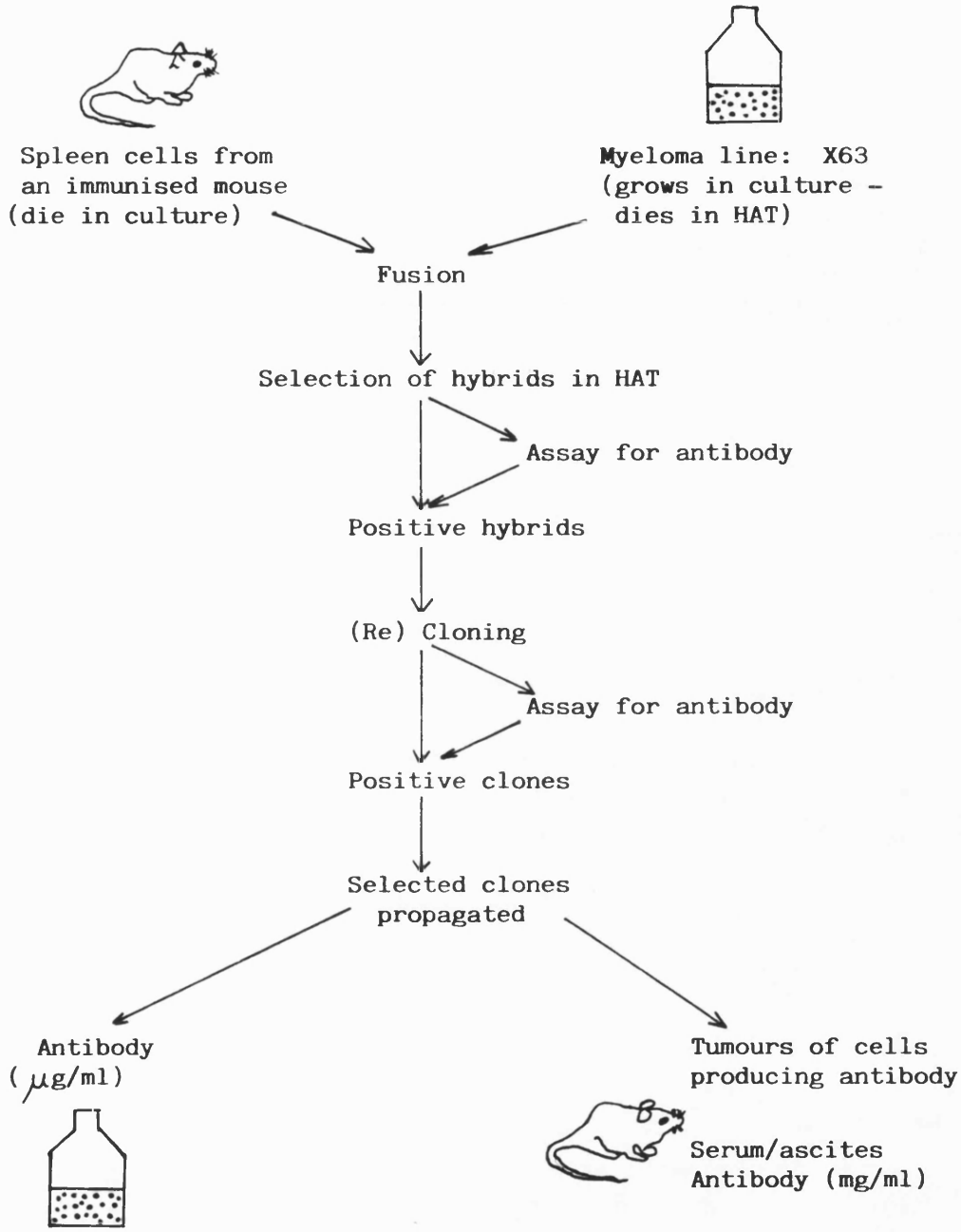
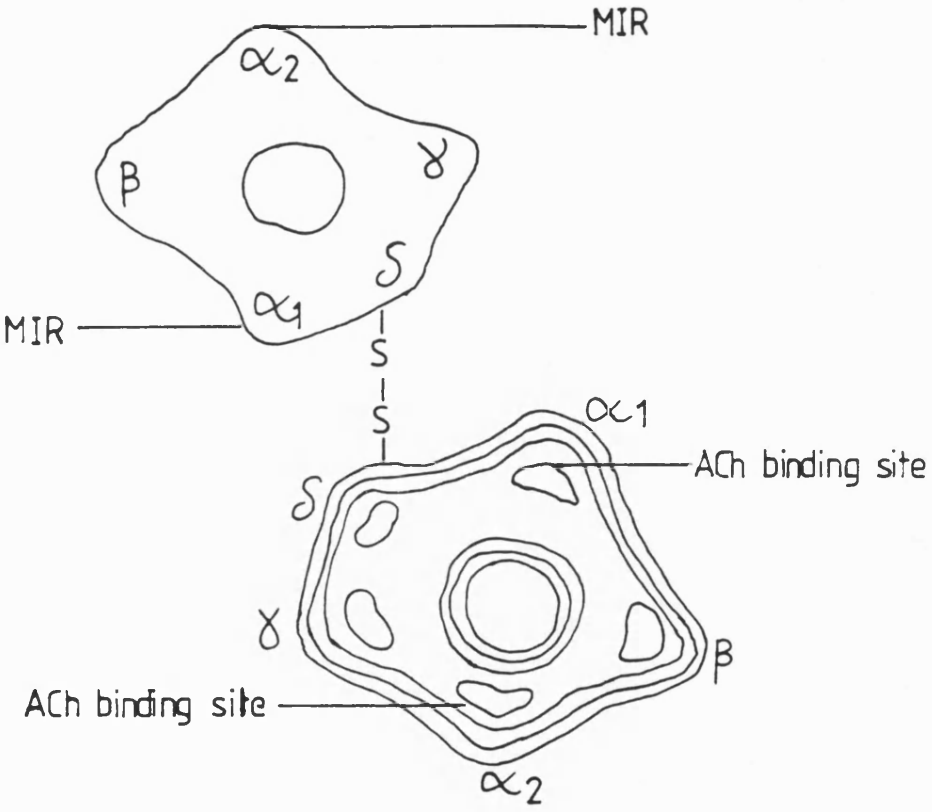


Figure 3 Location of subunits within the AChR from Torpedo Marmorata



The main immunogenic regions (MIR) are found on the extracellular surfaces of the α subunits and are on opposite sides of the receptor. The ACh binding site projects well within the molecular boundary of the structure and lies at the synaptic end of the subunit in agreement with the study of Kistler et al., 1982. (Taken from Kubalek et al., 1987).

The most critical and time-consuming stage in the production of Mabs is the screening of hybridomas, and the type of screening procedure used will dictate the types of Mabs ultimately produced. The methods which have been used include radioimmunoassay (RIA; Lindstrom et al., 1981), enzyme linked-immunosorbent assay, (ELISA; Dwyer et al., 1983) and a passive haemagglutination assay (Gomez et al., 1979).

Once obtained, the Mab must be characterised to be useful. Often this serves a dual purpose in that more is learnt about the receptor during the process of characterisation. The subunit specificities of Mabs can be determined by immunoprecipitation of [^{125}I]-labelled receptor subunits (Tzartos and Lindstrom, 1980; Tzartos et al., 1981 ; Gullick and Lindstrom, 1983) or by 'Western' blotting (Gullick and Lindstrom, 1983). The specificity can be more precisely defined against peptide fragments of [^{125}I]-labelled receptor subunits (Gullick and Lindstrom, 1983). Mab binding sites can also be mapped on native receptor by using competitive binding techniques (Tzartos and Lindstrom, 1980; Tzartos et al., 1981) and by analysis of the formation of complexes on sucrose gradients (Conti-Tronconi et al., 1981a).

The use of polyclonal and Mab probes in various aspects of AChR structure function and biosynthesis is described in the next sections. The specific contribution of Mabs as probes of AChR is summarised in Table 5, page 26.

Table 5 The contribution of Mabs to the understanding of the nAChR

INFORMATION GAINED	REFERENCE
Arrangement of nAChR subunits	Fairclough et al., 1983 Kubalek et al., 1987
Structural homology between receptor subunits	Tzartos and Lindstrom,1980 Tzartos et al., 1981 Froehner et al., 1983 Gullick and Lindstrom,1983
Structural homology between nAChRs of different species	Tzartos and Lindstrom,1980 Gomez et al., 1981 Mochly-Rosen and Fuchs, 1981 Tzartos et al., 1981 Tzartos et al., 1983
Receptor heterogeneity	examples : James et al., 1983 Mehraban et al., 1984 Souroujon et al., 1985 Whiting et al., 1986c
Transmembrane nature of nAChR	Froehner et al., 1983 Lindstrom et al., 1984 Sargent et al., 1984 Young et al., 1985 Ratnam et al., 1986 a and b (and references therein)
Means of purification of nAChR	Lennon et al., 1980 Momoi and Lennon, 1982, 1984
Mechanisms of receptor function	Souroujon et al., 1983 Donnelly et al., 1984 Mihovilovic and Richman, 1984 Wan and Lindstrom, 1985 Blatt et al., 1986 Fels et al., 1986
Biosynthesis of nAChR	Merlie et al., 1984
Nature of immuno-dominant region	Tzartos and Lindstrom,1981 Tzartos et al., 1981 Tzartos et al., 1983
Specificities of anti-(nAChR) antibodies in the sera of MG patients and in EAMG	Tzartos and Lindstrom,1981 Tzartos et al., 1982 Vincent, 1983 (for review)
Nature of neuronal nAChRs	see page 35 for details

3.1 Abs as probes of receptor structure

Mabs specific for the α, β, γ, δ subunits of receptor have been found and in addition, some Mabs have been found that cross-react with more than one subunit (Tzartos and Lindstrom, 1980; Tzartos et al., 1981 ; Gullick and Lindstrom, 1983; Froehner et al., 1983). This cross reactivity between subunits by Mabs provided evidence for structural homology of receptor subunits within the same species. Additionally, Mabs have demonstrated homology between AChR subunits from various species (Table 5).

Both polyclonal antibodies and Mabs have demonstrated the extent of similarity among AChRs from various species (Lindstrom et al., 1978ab; Tzartos and Lindstrom, 1980; Gomez et al., 1981; Mochly-Rosen and Fuchs, 1981; Tzartos et al., 1981 ; Tzartos et al., 1983). Many Mabs are species-specific, others cross-react with a limited number of species and some bind to all species tested. Greatest cross-reactivity was observed by anti-α subunit Mabs (Tzartos and Lindstrom, 1980; Tzartos et al., 1981 , 1983).

Competitive binding techniques (Tzartos and Lindstrom, 1980; Tzartos et al., 1981) have shown that the majority of antibodies (both polyclonal and Mab) raised against native receptor are directed against a small immunodominant region or main immunogenic region (MIR) on the receptor. Several studies have shown that the MIR is a conformationally-dependent antigenic determinant (Tzartos and Lindstrom, 1980; Tzartos et

al., 1981). The MIR is located on the extracellular surface of α-subunits but is distinct from the ACh binding site and the carbohydrate moiety (Tzartos and Lindstrom, 1980; Gullick et al., 1981). The MIR is present on all species examined to date and so is conserved through evolution although its function is not understood. Peptide mapping experiments have localised the MIR to between α residues 46 and α 127 (Ratnam et al., 1986a). More recently Tzartos and co-workers (1988) have more precisely defined the MIR region in human AChR to α-residues 67-76, using Mabs to synthetic peptides of the α-subunit of human AChR.

A corollary of using Mabs to detect similarities between species is that they can be used to detect differences (for example see James et al., 1983). Indeed, Mabs have provided evidence for immunological differences between types of receptor, namely, J and EJ forms of AChR (see Introduction, page14) (Mehraban et al., 1984; Souroujon et al., 1985; Whiting et al., 1986c).

The findings of structural homology and dissimilarity by Mabs and polyclonal antisera were confirmed by cDNA sequencing of the receptor subunits (see Introduction, page15).

3.2 Abs as probes of the transmembrane disposition of the nAChR

Early studies using polyclonal anti-(AChR) serum (Tarrab-Hazdai et al., 1978) and proteolytic degradation (Strader and Raftery, 1980; Wennogle and Changeux, 1980; Anderson and Blobel, 1981; Wennogle et al., 1981) provided evidence that AChR is a transmembrane protein. Studies using anti-(AChR) Mabs have confirmed these findings and shown that all five subunits are

exposed in the cytoplasm (Froehner et al., 1983; Lindstrom et al., 1984; Sargent et al., 1984; Young et al., 1985).

The cellular positions of the amino (N-) and carboxy (C-) termini are important in predicting the subunit folding pattern in the lipid bilayer, however this area of work remains contentious. Studies on the biosynthesis of AChR (Anderson and Blobel, 1983) and using domain specific Mabs have provided evidence that the N-terminus is located extracellularly (Neumann et al., 1984). The location of the C-terminus, however, is still a subject of debate. Immunological evidence, using domain specific Mabs (Lindstrom et al., 1984; Young et al., 1985; Ratnam et al., 1986b) and a model based on the primary sequence structures of the subunit polypeptide chains (Finer-Moore and Stroud, 1984) have suggested that the C-terminus is located cytoplasmically. Hence, indicating that the N- and C- termini are on oppsite sides of the membrane and that an odd number of membrane spanning polypeptide regions exist. A more recent study by McCrea and co-workers (1987) has demonstrated that the $\delta-\delta$ disulphide bridge of AChRs from Torpedo electric organ is extracellular, a finding most easily reconciled with an extracellularly located N- and C- termini and hence an even number of transmembrane crossing per subunit. These findings are in agreement with the early working models proposed by Claudio et al., (1983); Devilliers-Thiery et al., (1983) and Numa et al., (1983). The difficulty of accurately predicting the tertiary structure of the nAChR is highlighted by these conflicting results.

3.3 Abs as probes of the orientation of the α-subunits within receptor monomers

The order of the AChR subunits around the ion channel is fundamental in understanding the mechanism of action of the receptor. The orientation of α-subunits within the receptor complex has been determined by using anti-(AChR) Mabs (Fairclough et al., 1983; Kubalek et al., 1987) and α-BGT (Holtzman et al., 1982; Oswald and Changeux, 1982; Kubalek et al., 1987) as probes. There is consensus that the two α-subunits are diametrically opposed (Zingsheim et al., 1982; Kubalek et al., 1987). A recent representation of the arrangement of the receptor subunits is given in Figure 3 (page 24).

3.4 Abs as probes of receptor function

Affinity-purified AChR can be reconstituted into model membranes and can retain function with ion channels similar to those observed in intact muscle (Anholt et al., 1984). These model receptor systems have proved useful for the analysis of the effects of antibody binding on ion channel activity. Mabs which bind at or near the ACh binding site and block AChR function have been reported (Souroujon et al., 1983; Mihovilovic and Richman, 1984). Additionally, Mabs which are specific for the cytoplasmic domains of the AChR have been shown to inhibit function (Blatt et al., 1986) and these Mabs have directly implicated the β and δ subunits in ion-channel gating.

3.5 Abs as probes of nAChR biosynthesis

Both polyclonal and Mabs raised against the subunits of AChR have made it possible to identify newly synthesised receptor subunits. Specific antibodies were shown to immunoprecipitate all four nascent chains of newly-synthesised AChR, translated by Torpedo mRNA in cell free proteins synthesising systems (Mendez et al., 1980; Anderson and Blobel, 1981) and in microinjected Xenopus ooctyes (Sumikawa et al., 1981). A study by Anderson and Blobel (1981) demonstrated that all four subunits of Torpedo receptor are synthesised as individual polypeptides. However, cell-free translation of mRNA coding for the four receptor subunits does not lead to the binding of α-BGT or to the assembly of the resulting subunits to the $\alpha 2, \beta, \gamma, \delta$, complex and this has limited their use. The use of the muscle cell line BC3H-1 has overcome these limitations. Radiolabelled subunit-specific Mabs have been employed in this system to establish a time course for the processes leading ultimately to the localisation of the receptor in the membrane. This sequence involves synthesis ($\sim$1min), (maturation resulting in ability to bind α-BGT and conformationally dependant Mabs; 1-30min), assembly (30-90min) and transport (90-150min). A detailed account of AChR biosynthesis is given by Merlie et al., (1984).

3.6 Studies of neuronal nAChRs using Mabs

nAChRs are known to occur not only at neuro-effector junctions

in electric tissue and skeletal muscle but within the autonomic nervous system, on chromaffin cells, in the gut, the spinal cord and the brain. Early attempts to identify these receptor sites were based on the use of α-BGT (Schmidt et al., 1980). However, the failure of this toxin to antagonise nicotinic responses in the mammalian CNS and autonomic nervous system (Schmidt et al., 1980) has called into question its suitability as a probe for the neuronal receptor. Mabs have been used as alternative probes in the identification, localisation and characterisation of putative nicotinic receptors in neurons. The picture of nAChRs in the central nervous system (CNS) is complicated and it appears that the CNS may contain AChRs that bind α-BGT and AChRs that do not. Such findings suggest a diverse family of AChRs and the evidence behind these findings will be discussed briefly.

Early studies by Patrick and Stallcup in 1977 demonstrated the presence of functional AChRs which did not bind α-BGT and also of non-functional α-BGT-binding proteins. α-BGT binding proteins have been reported in some instances purified from various CNS sources (Oswald and Freeman, 1979, 1981; Norman et al., 1982; Wonnacott et al., 1982; Dolly and Barnard, 1984; Conti - Tronconi et al., 1985; Whyte et al., 1985; Whiting and Lindstrom, 1987a). The central α-BGT binding protein is similar to peripheral AChR (Barnard and Dolly, 1982) and shows limited immunological cross-reactivity with anti-(peripheral nAChR) antibodies (Norman et al., 1982; Wonnacott et al., 1982;

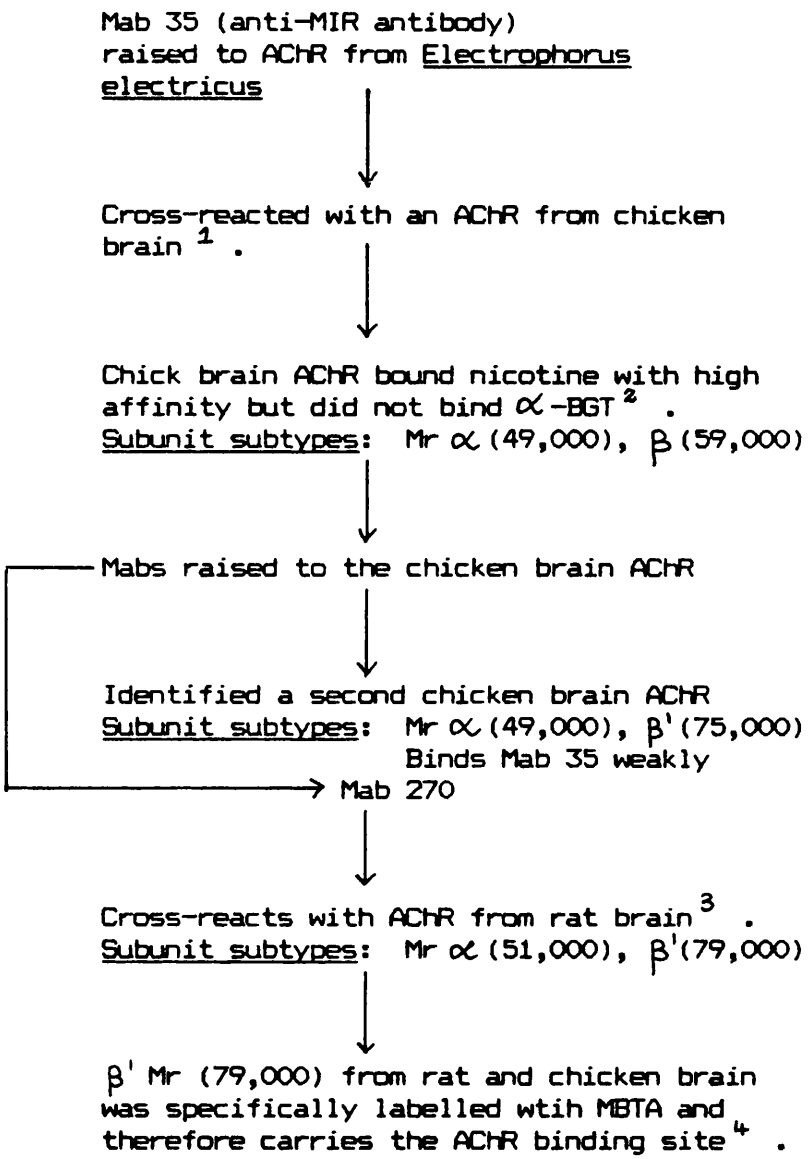
Mehraban et al., 1984; Mills and Wonnacott, 1984).

Analysis of the subunit composition of toxin affinity-purified central receptor from chick optic lobe has revealed three (Conti-Tronconi et al., 1985) and more recently four subunits (Barnard et al., 1986). The toxin binding component isolated from rat brain also comprised four subunits (Whiting and Lindstrom, 1987a). These latter studies, imply conservation of the $\alpha 2, \beta, \gamma, \delta$ subunit stoichiometry of peripheral receptors (see Figure 4, page 35).

An alternative probe of neuronal nicotinic receptors has been radiolabelled nicotine. Using this ligand, high affinity nicotine binding sites were separated from α-BGT bindings sites in an affinity purification scheme (Wonnacott, 1986; Whiting and Lindstrom, 1986a, b). These studies add to the evidence provided by pharmacological comparisons (Marks and Collins, 1982; Rapier et al., 1985) and regional distribution studies (Clarke et al., 1985) that the α-BGT and high affinity nicotine binding sites in mammalian brain are separate functional entities. The significance of this is not understood. Purification of the nicotine binding protein has been attempted using the 6-hydroxyethyl (Abood et al., 1983) and 6-hydroxymethyl (Abood et al., 1987) derivatives of nicotine as affinity ligands. A successful approach has been the use of anti-(AChR) Mabs as affinity ligands and this has been exploited by Whiting and Lindstrom in the isolation, purification and

characterisation of neuronal AChR subtypes from chicken and rat brain (Whiting and Lindstrom, 1986a, b, 1987a, b, see Figure 4, page 35). However, one disadvantage of using Mabs as affinity ligands is that the protein obtained is in a non-functional form. In spite of this, an insight into the structural heterogeneity of neuronal AChR subtypes has been gained. Two AChR subtypes, $\alpha\beta$ (from chicken) and $\alpha\beta'$ (from rat) have been isolated. These subtypes were found to have identical affinity for (-)-nicotine. Their α-subunits are similar or identical and share some antigenic determinants with α-subunits from AChRs of electric organ and muscle. These subtypes, $\alpha\beta$, and $\alpha\beta'$, are however, distinguishable by their β subunits, which have different Mr values (see Figure 4, page 35). In the case of neuronal receptors the ACh binding site has been localised to the larger molecular weight subunit rather than the lowest molecular weight subunit (α) in the muscle nAChRs. Recently an anti-(MIR) antibody generated against Torpedo electric organ has been used to localise, histochemically, neuronal nAChRs in rat brain. The areas stained with this antibody paralled those areas of the brain exhibiting [^{3}H]-nicotine binding (Deutch et al., 1987).

Fig 4 Use of Monoclonal Antibodies in the Study of Central nAChRs



1. Swanson et al., 1983
2. Whiting and Lindstrom, 1986a, b
3. Whiting and Lindstrom, 1987a
4. Whiting and Lindstrom, 1987b.

4. The involvement of AChR in myasthenia gravis

Myasthenia gravis (MG) is an autoimmune disease characterised by muscle weakness and rapidly fatiguing neuromuscular transmission (Simpson, 1960).

4.1 The neuromuscular junction in MG

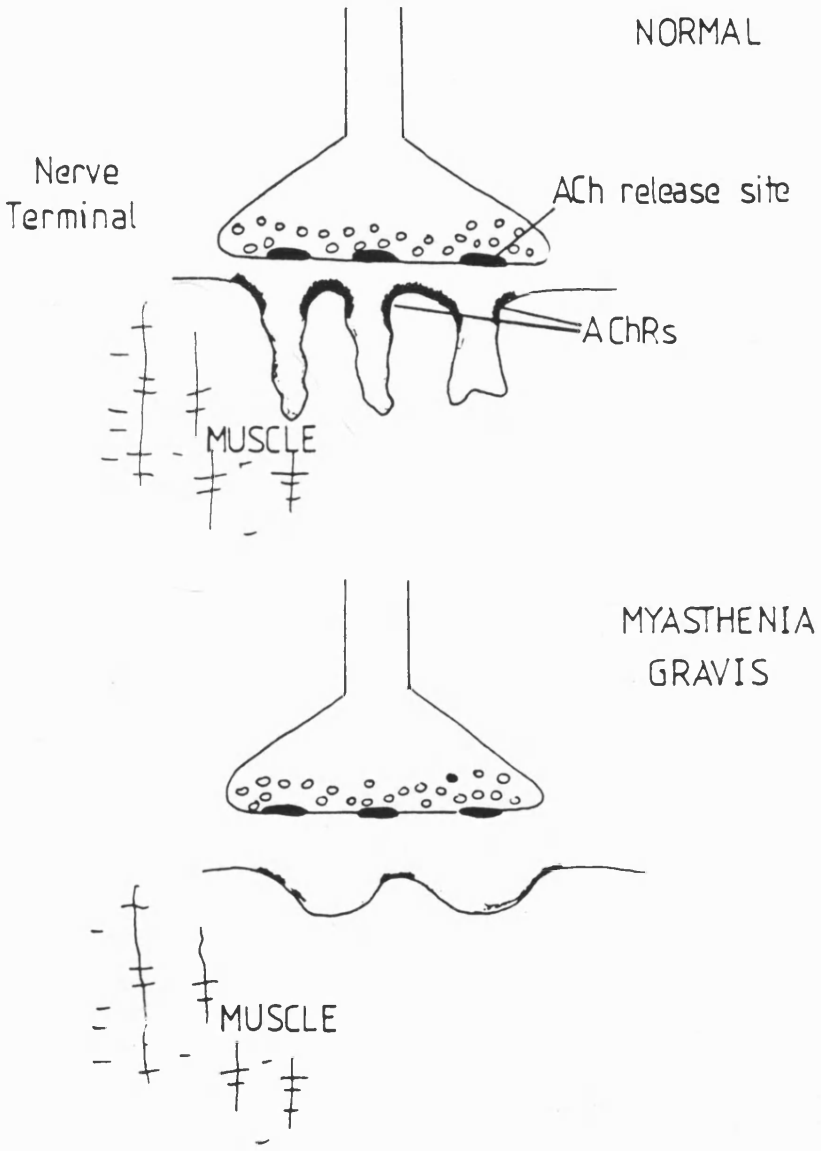
A defect at the post-synaptic membrane was implied by a number of findings: the post-synaptic membrane of MG patients appears highly simplified at the EM level and binds less iodinated α-BGT than normal control post-synaptic membranes (Fambrough et al., 1979). This latter observation directly suggested the involvement of the nAChR in the disease. The various physiological changes at the NMJ of myasthenic patients (see Figure 5, page 37) result in decreased efficiency of synaptic transmission, which if reduced to below threshold values, will result in failure to trigger an action potential and muscle contraction.

4.2 The autoimmune nature of MG

The association of MG with other autoimmune diseases, the occurrence of thymic abnormalities in a high percentage of MG patients and the beneficial effects of thymectomy and steroid therapy were several factors pointing to an autoimmune origin in MG (see Harrison and Behan, 1986).

Evidence for an autoimmune response directed against AChR was provided by Patrick and Lindstrom (1973) when they showed that

Figure 5 Comparison of normal and myasthenic neuromuscular junctions



The MG junctions show a normal pre-synaptic terminal, a simplified post-synaptic membrane with reduced numbers of AChR, sparse shallow folds where complement-mediated focal lysis results in shedding of membrane fragments (containing AChR and C3) into the synaptic cleft, and antigenic modulation, involving antibody cross-linking of AChR, internalisation and proteolysis. The synaptic space is widened as a result of the loss of membrane.

immunisation of rabbits with purified nAChR produced an experimental disease (Experimental Autoimmune Myasthenia Gravis; EAMG), with many similarities to MG.

The animal model has proved useful for the study of MG and possible immunotherapy (for review on EAMG see Harrison and Behan, 1986). This led to the discovery by Almon et al., (1974) that immunoglobulin, from some myasthenic patients could inhibit α-BGT binding to AChRs in detergent extracts of rat muscle. Following the development of a more sensitive radioimmunoassay AChR-binding antibodies were detected in 87% of patients with MG (see Lindstrom, 1979; Lindstrom et al., 1981).

4.3 The pathogenic role of anti-(AChR) antibodies

The key role of circulating anti-(AChR) antibodies in the pathogenesis of MG has been inferred from a number of studies. In EAMG, myasthenic symptoms can be produced by passive transfer of IgG from myasthenic patients (Toyka et al., 1975, 1977), antibodies from myasthenic animals (Lindstrom et al., 1976) or Mabs (eg: Lennon and Lambert, 1980). Placental transfer of anti-(AChR) antibodies gives rise to neonatal MG (Keesey et al., 1977). Immunoglobulin and the C3 complement component are found at the degenerating myasthenic endplate (see Engel, 1987). Plasma exchange can also cause a temporary improvement in muscle strength in MG, paralled by a fall in serum anti-(AChR) antibody levels (Dau et al., 1977).

Circulating anti-(AChR) antibodies play a key role in reducing the number of effective receptors at the myasthenic endplate.

The mechanisms involved are similar in MG and EAMG and can be divided into three broad categories :

1. Direct blockage of AChR

   Direct blockade of receptor sites by antibody has been shown to occur in skeletal muscle cultures (Drachman et al., 1977; Fulpuis et al., 1981). Myasthenic immunoglobulins have also long been known to block binding of [^{125}I]-α-BGT to detergent solubilised AChR (Almon et al., 1974; Lennon, 1976; Vincent and Newsom-Davis, 1979).

2. Effect on Turnover of AChR

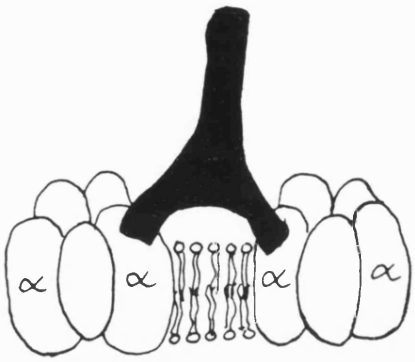
   An increase in receptor degradation rate can be shown both *in vitro* and *in vivo*. It is triggered by antibody alone and is dependent upon the ability of IgG to cross-link receptors (Drachman et al., 1978; Conti-Tronconi et al., 1981b) and the divalent nature of the antibodies (see Figure 6, page 40).

   Morphological studies indicate that such cross-linking causes re-distribution of AChR in the membrane followed by enhanced endocytosis.

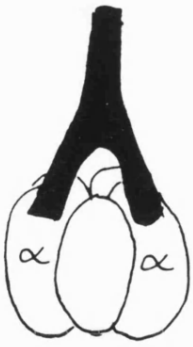
3. Complement-dependent membrane lysis

   The evidence for the complement-mediated destructive effect of anti-(AChR) antibodies is mainly indirect. IgG, C3 and the final lytic component, C9, have been found at the myasthenic endplate and degenerated membrane fragments found in the synaptic space of animals with EAMG and in MG patients. An excellent review of these studies is given by

Figure 6 Inter and intra-molecular cross-linking of AChRs by anti-AChR antibodies against the α-subunits.



INTERMOLECULAR
CROSS-LINKING



INTRAMOLECULAR
CROSS-LINKING

Engel (1987). Recently work by Childs et al., (1985) has demonstrated directly the lytic effects of anti-(AChR) antibodies on rat muscle cells, with 9 of 13 myasthenic serum samples showing complement-dependent myolytic activity.

4.4 Cellular immunity in MG

As in all autoimmune diseases, the immune system in MG responds to a 'self' antigen in an inappropriate way,. This may be due to: (a) an alteration in the antigen causing it to be perceived as 'non-self'; (b) defective control of the immune system allowing it to respond to a normal antigen or (c) a combination of both. Examining point b, it is conceivable that proliferation of autoantibody-forming B-cells could result from either a decrease in the number and/or function of suppressor T-cells, or an increase in the number and/or function of helper -T cells.

Hence, many workers have sought evidence for abnormalities in the cellular immune system in MG by comparing the proportions of B and T lymphocytes in MG patients with those of normal controls and by studying in detail the role of T-suppressor (Ts) and T-helper (Th) cells in MG by functional and cell surface marker assays (reviewed by Lisak et al., 1985: Harrison and Behan, 1986). While the results are conflicting, the majority of reports point to increased B-cell and decreased T-cell populations in both the thymus and peripheral blood in MG.

The importance of regulatory T-cell function in MG is inferred from the fact that susceptibility to MG is associated with the MHC antigens (Oosterhuis et al., 1981) and several experiments support the concept that AChR-specific T cells are important in the aetiology and immune regulation in MG. Peripheral blood lymphocytes (PBL) are sensitised against AChR in MG, and undergo proliferation when incubated with AChR from electric eel (Abramsky et al., 1975; Richman et al., 1976, 1979) and Torpedo electric organ (Conti-Tronconi, 1977, 1979, 1982; Hohlfield et al., 1984). Proliferative responses of PBL from MG patients and controls to human AChR peptides have also recently been studied (Harcourt et al., 1987; Hohlfeld et al., 1987).

The T-cell recognition site(s) on the AChR have not been as clearly defined as the antibody-binding sites. One approach to this problem has been through the isolation of T-cell lines specifically reactive to the AChR (see Harcourt and Jermy, 1987 for brief review). Indeed T cell lines have been produced which are specific for Torpedo AChR (Hohlfeld et al., 1984; Fujii and Lindstrom, 1987). However, due to the requirement of large amounts of AChR protein needed to assay and maintain such cell lines, T cell lines specific to human AChR have proved difficult to obtain, although the use of synthetic peptides corresponding to human AChR may circumvent this problem (Fujii and Lindstrom, 1987). Nevertheless, it is proving possible to locate important T-cell recognition sites of T cell lines by using synthetic peptides corresponding to the human α-subunit in proliferative assays (see Harcourt and Jermy, 1987). It appears that the

immunodominant T-cell recognition sites lies on the same subunit as the MIR but is distinct from this important antibody binding site (Hohlfeld et al., 1987).

An alteration of immunoregulation seems likely to be necessary for the maintenance of the autoimmune response in MG, however the origin of the autoimmune response in unknown. The possibility of it being initiated by an external factor such as a virus or bacterial infection has been studied. Stefansson et al., (1985) discovered that AChR cross-reacts with proteins of Escherichia coli, Proteus vulgaris and Klebsiella pneumoniae and proposed that this cross-reactivity may be the cause of an immune attack directed against AChR. However, the average spectrum of antireceptor antibody specificities is the same in MG patients and in rats immunised with purified receptor (Tzartos et al., 1982) suggesting that the immunogen in MG is intact human receptor rather than a single cross-reacting determinant on a bacterium or virus. There is however considerable evidence that the autoimmune reaction in MG may originate within the thymus gland.

4.5 The thymus in MG

The association between the thymus and AChR is of special interest in view of the involvement of the thymus in MG. A high incidence of thymic abnormalities are associated with MG (Thomas et al., 1982; Bofill et al., 1985) and thymectomy is beneficial in many MG patients (Oosterhuis, 1981; Hankins et al., 1985).

Changes in thymic lymphocyte populations have also been reported in MG (reviewed by Lisak et al., 1985; Harrison and Behan, 1986). The presence of AChR in the thymus has been demonstrated by a number of methods, including the use of anti-(AChR) antibodies.

Thymus cells, that have many histological characteristics of striated muscle were initially described by Mayer in 1888. These cells, later named 'myoid cells' (Hammer, 1905) show cross-reactivity with anti-skeletal muscle antibodies (Van de Geld, 1966). More recently, cells that are typical of skeletal muscle cells in culture have been cultured from thymuses of rat (Kao and Drachman, 1977; Wekerle et al., 1975), mice (Wekerle et al., 1981) and humans (Kao and Drachman, 1977). These cells express surface AChRs.

A recent study by Kirchner et al., (1987) using anti-AChR specific Mabs has reported the presence of AChR epitopes on thymoid cells from myasthenic and non-myasthenic individuals. Extracts of thymus tissues from a wide variety of vertebrate species also contain AChR (Lindstrom et al., 1976; Ueno et al., 1980; Raimond et al., 1984). Immunological cross-reactivity between a thymic component and anti-(AChR) antibodies has been reported (Aharanov et al, 1975).

Both thymic epithelial cells (Engel et al., 1977, Matsumoto et al., 1986; Kirchner et al., 1987) and thymic lymphocytes (Fuchs et al.,1980; Horvat et al., 1983; Pizzighella et al., 1983b; Riviera et al., 1987) have been reported to bear AChR. The latter studies employed antibodies against the nAChR to detect

cross-reactivity by immunofluorescence (Fuchs et al., 1980; Horvat et al., 1983; Pizzighella et al, 1983b; Riviera et al., 1987) and radioimmunological techniques (Fuchs et al., 1980). One Mab, raised against the ACh binding site of the receptor, was found to interfere with the inhibitory effect of succinylcholine on mouse thymocyte proliferation and cyclic AMP production (Pizzighella et al., 1982), directly implicating a role for cholinergic ligands modifying cellular functions of thymocytes through the presence of a functioning membrane AChR. Thymic cells cultured from MG patients are capable of producing anti-(AChR) antibodies (see Table 6, page 46) and can also selectively enhance the production of anti-AChR antibodies from PBL in vitro (Vincent et al., 1978; Newsom-Davis et al., 1981a, b; Willcox et al., 1984). It has been suggested that the enhancing cell type is a specific antigen-presenting cell (Willcox et al., 1984). A cell line producing anti-(AChR) Mabs has been established from the thymus of a MG patient (Kamo et al., 1982).

4.6 AChRs on peripheral blood lymphocytes

The presence of muscarinic AChRs on lymphocytes has been well established (Dulis et al., 1979; Gordon et al., 1978; Lopker et al., 1980; Zalcman et al., 1981; Atweh et al., 1984; Adem et al., 1985, 1986b) by direct binding studies. There are however indications that AChRs with nicotinic properties are also present on lymphocytes and this may represent further diversification in the family of nAChRs already described. Many of the studies used to identify this receptor have examined the

Table 6 Reports of Anti-AChR antibody synthesis by peripheral blood and thymic lymphocytes from MG patients

Anti-AChR synthesis by:	Reference
Cultured blood lymphocytes	Clarke et al., 1979 Vincent et al., 1979 Newsom-Davis et al., 1981a Lisak et al., 1983a, 1984 Willcox et al., 1984 Fujii et al., 1986 Kaufman and Oger, 1987
Thymic lymphocytes	Vincent et al., 1978,1979 Ohta et al., 1980 Newsom-Davis et al.,1981a Scadding et al., 1981 Lisak et al., 1983b Willcox et al., 1983,1984 Fujii et al., 1984,1985, 1986
Monoclonal anti-(AChR) antibody secreting cell line from MG thymus	Kamo et al., 1982
Increased production of anti-(AChR) synthesis of PBL by addition of irradiated autologous thymic cells	Newsom-Davis et al., 1981a, b Willcox et al., 1984

effect in vitro of cholinergic ligands on PBL proliferation (Richmon and Arnason, 1979; Richman et al., 1981; Ménard and Rola-Pleszczynski, 1983). Other studies have described an inhibitory role for carbachol in E-rosette formation (Mizuno et al., 1982a, b). These latter studies suggested that nAChRs may be present on a subset of human T cells.

Direct binding of iodinated α-BGT has de monstrated specific binding sites on PBLs from myasthenic patients which are not present on normal control PBL (Morrell, 1979, 1981). Specific binding sites for [^{3}H]-nicotine have also been found on normal human lymphocytes (Davies et al., 1982; Adem et al., 1985, 1986a) and human granulocytes (Davies et al., 1982; Hoss et al., 1986).

The precise function of nAChRs on PBLs in unknown. Whaley et al., (1981) demonstrated that C2 synthesis by human monocytes is modulated by a nAChR, whereas Máslinski et al., (1980) described a nicotinic receptor on rat lymphocytes that may be involved in ACh transport.

The possible presence of AChR on PBL has important implications in MG. It could act as a potential autoimmunogen but also as a target for circulating anti-(AChR) antibodies. Studies demonstrating immunological cross-reactivity of anti-(AChR) antibodies with a nicotinic receptor on lymphocytes have been limited. Immunological cross-reactivity of myasthenic sera and a component on normal human PBL has been reported (Mischak and Dau, 1981) and other workers (Shimizu, 1980; Arimori et al.,

1981) have observed changed membrane ultrastructure and micro-viscosity in peripheral T lymphocytes from myasthenic patients: changes which can be reproduced *in vitro* by addition of myasthenic serum to normal lymphocytes. Further work examining the nicotinic receptor on PBL and possible immunological recognition by anti-(AChR) antibodies, using Mabs as specific probes, may help to clarify this situation.

Aims of the Project

Both polyclonal and monoclonal antibodies have proved invaluable tools for the study of nAChR from various tissue and species sources. Workers have used these antibody probes in the study of novel receptor types of the central nervous system where the receptor content is smaller and pharmacologically distinct from that at the neuromuscular junction. nAChRs have also been implicated to be present on lymphocytes and the presence of this receptor is of relevance in the disease myasthenia gravis. Studies of this receptor type have been limited in comparison to other receptor types, hence this thesis is concerned with the examination of this novel receptor.

The thesis is divided into five sections. The first deals with the purification and characterisation of nAChR and the second and third, the production and characterisation of polyclonal and monoclonal antibodies against this receptor. The final two sections concern the study of nAChR on lymphocytes with the prepared antibody probes and with respect to cholinergic binding sites.

MATERIALS

1. Sources of AChR

Torpedo marmorata electric organs were purchased from Institut de Biologie Marine, Arcachon, France and stored at -80°C.

Bovine fetal calves were obtained fresh from the local slaughter house. Muscle from the fore and hind limbs were removed and frozen in liquid nitrogen before being stored at -80°C for up to three months.

Human adult muscle was supplied by local hospitals and was obtained from lower limb amputations resulting from severe vascular disorders. Within 15 minutes of amputation, calf muscle was dissected free from fat, tendon and skin and immediately frozen in liquid nitrogen and stored at -80°C for up to two months.

2. Immunoreagents

Blood samples were obtained from normal volunteers within the department. Myasthenic blood samples were obtained from local hospitals. All blood samples were collected with 10IU heparin per ml of blood.

Goat anti-(human IgG), Goat anti-(rabbit IgG), Rabbit anti-(BSA) and Rabbit anti-(human Fab$_\mu$) antisera were gifts from colleagues.

Rabbit anti-(mouse IgG) and Goat anti-(mouse IgG) antisera were prepared as described in Methods, Section B. Rabbit anti-

(fetal calf AChR) antisera were prepared as described in Methods, Section B.

Rabbit anti-(Torpedo AChR) antisera was a kind gift from Dr Wonnacott.

Mouse anti-neuro filament and mouse anti BSA ascites fluid were gifts from colleagues.

Rabbit IgG coated ACA beads were a kind gift from Dr A Jehanli.

Protein-A conjugated Sepharose 4B beads were from Pharmacia Ltd., Hounslow, UK.

Normal mouse and normal rabbit sera were prepared from animals obtained from the University's animal house.

Sheep red blood cells were obtained in Alsever's solution from Tissue Culture Services, Slough, UK.

Freund's complete and incomplete adjuvants were from Miles Lab., Stoke Poges, Slough, UK.

Enzyme-conjugated second antibodies and substrate reagents for ELISA studies were obtained from Sigma Chemical Company, Poole, Dorset, UK.

ELISA plates were obtained from NUNC, Roskilde, Denmark.

3. Tissue Culture Reagents

RPMI-1640, Modified Eagles Medium, fetal calf serum, donor horse serum, antibiotics, selection media for Mab production and

Ficoll-Hypaque separation medium were obtained from Flow Labs, Irvine, UK.

Tissue culture plates and flasks were obtained from Nunc, Roskilde, Denmark.

Mouse myeloma cell line X63 - Ag8. 6. 5. 3. was a gift from Dr K. Thompson.

Collagen prepared from rat tails was kindly provided by J. Ward (protein concentration 0.75 mg/ml).

4. Counting Instruments

[^{125}I] was counted in a LKB 1280 Ultragamma counter.

[^{3}H] was counted in a Packard Tri-carb scintillation counter (Model 3255).

5. Radiochemicals

All radiochemicals were obtained from Amersham International (Amersham, Bucks., UK). Carrier free Na[^{125}I] in dilute NaOH (100mCi/ml) was stored at room temperature and was used within three weeks of its activity reference date.

1-quinuclidinyl-(phenyl - 4 ^{3}H) benzilate ([^{3}H]-QNB) (46Ci/mmol) was stored at -20°C.

(-)-[N-methyl- ^{3}H]-nicotine ([^{3}H](-)-nicotine) (60-85Ci/mmol) was stored at -20°C.

Optiphase (Safe) scintillation fluid was obtained from LKB Pharmacia, Milton Keynes, Bucks., UK.

6. Ligands

α-BGT from Bungarus multicinctus was purchased from Boehringer Corp., Mannheim, W. Germany, as a lypholised powder.

Naja naja siamensis venom was obtained from Miami Serpentarium, Florida, U.S.A.

Benzoquinonium chloride was a generous gift from Stirling Winthrop Inc., Rensselaer, N.Y., U.S.A.

Atropine, d-tubocurarine,. dihydro-β-erythroidine, hexamethonium, carbachol, acetylcholine, decamethonium, nicotine hydrogen (+) tartrate, nicotine (-) ditartrate, 1-1-dimethyl-4-phenyl piperazinium iodide and anti-acetylcholinesterase inhibitors physostigmine and BW 284C51 were kind gifts from colleagues.

7. Chemicals

Standard laboratory reagents were obtained from Sigma Chemical Company, Poole, Dorset, UK. BDH Chemicals, Kingston-upon-Thames or Aldrich Chemical Company Ltd., Gillingham.

"Aged" chromic chloride was a kind gift from Dr A Jehanli.

Gel filtration reagents were supplied by Pharmacia Ltd., Hounslow, U.K.

Ion-exchange resins, DEAE cellulose filter discs, GFB and GFC glass fibre discs were obtained from Whatman Laboratory Sales Ltd., Maidstone, Kent, UK.

Nitrocellulose paper and Amicon concentrators were from Millipore, Molsheim, France.

Polyethylenimine and lactoperoxidase was obtained from Sigma Chemical Company, Poole, Dorset, UK.

SECTION A

PURIFICATION AND DETECTION OF AChR

METHODS

1. Preparation of [^{125}I]-labelled-α-BGT

1.1 Iodination of α-BGT

1.2 Determination of the biological activty of [^{125}I]-α-BGT

2. Assay of AChR activity

2.1 Detergent soluble AChR

2.1a Ammonium sulphate precipitation method
2.1b Polyethyleneimine-treated GFC filtration method

2.2 DEAE-cellulose filtration method

3. Purification of AChR

3.1 Extraction procedure
3.2 Affinity chromatography

3.2.1 Purification of α-toxin from the venom of *Naja naja siamensis*
3.2.2 Preparation of the α-toxin affinity resin
3.2.2a CNBr activation of Sepharose 4B
3.2.2b Coupling of the α-toxin to activated Sepharose 4B

3.3 Adsorption of AChR from detergent extracts

3.4 Elution of AChR from the α-toxin beads

3.4.1 Using carbamylcholine
3.4.2 Using benzoquinonium chloride

3.5 Determination of the specific activity of AChR preparations

3.6 Protein estimation

4. Characterisation of purified AChR

4.1 SDS-PAGE

4.2 Gel filtration of *Torpedo* AChR

1. Preparation of [^{125}I]-labelled α-BGT

1.1 Iodination of α-BGT

α-BGT was iodinated with ^{125}I by the chloramine-T method (Hunter, 1967) as modified by Urbaniak et al., (1973). The procedure was carried out at room temperature and unless otherwise stated, all solutions were made up in 50mM potassium phosphate buffer, pH 7.4

Carrier-free Na [^{125}I] in dilute sodium hydroxide (100mCi/ml, 20μL) was added to α-BGT (2.5nmoles, 4μL). To start the reaction, chloramine T (0.5% w/v, 20μL) was added. After constant stirring for 1 min the reaction was terminated by the addition of 0.016% (w/v) sodium metabisulphite (1.5 ml), followed by 1% (w/v) potassium iodide (400μL).

The labelled protein was separated from free [^{125}I] by passage through a Sephadex G-25 column(20cm x 1cm) pre-equilibrated in 10mM potassium phosphate buffer, pH 7.4, containing 1% (w/v) BSA. Labelled protein was eluted with the same buffer. Fractions (20 x 1ml) were collected and samples (5μL) were counted for radioactivity. The peak fractions containing [^{125}I]-α-BGT were pooled, stored at 4°C and used within 3 weeks.

The specific radioactivity of the [^{125}I]-α-BGT was calculated assuming 100% recovery of protein.

1.2 Determination of the biological activity of [^{125}I]-α-BGT

The biological activity of the [^{125}I]-α-BGT was determined by measuring the proportion that could be bound by a large excess of purified Torpedo marmorata AChR in the absence and presence of an excess of the competitive antagonist BZQ. Triplicate samples of Torpedo AChR (20pmol, 100μL) were incubated with [^{125}I]-α-BGT (0.2pmol, 50μL) for 90 min at 23°C in the presence and absence of BZQ (25mM, 50μL), followed by filtration through DEAE-cellulose filters, as described in Section A 2.2.

2. Assay of AChR activity

All the assays described involve the incubation of AChR with an excess of [^{125}I]-α-BGT. Non-specific binding was determined by carrying out parallel incubations in the presence of a large excess of BZQ (2.5mM, 50μL). Assay totals consisted of [^{125}I]-α-BGT (2.5-4.0nM, 50μL) alone. Assay blanks, to determine the non-specific binding of radioligand to the filters, comprised [^{125}I]-α-BGT and buffer.

2.1 Detergent soluble AChR

AChR was assayed in 'crude' detergent extracts of muscle or electric organ by two procedures.

2.1.a Ammonium sulphate precipitation method

This procedure is adapted from that described by Meunier et al., (1972).

Triplicate samples of the receptor (100µL) were incubated (45 min, 23°C) with [^{125}I]-α-BGT (2.5-4.0nM, 50µL) in extraction buffer, comprising 10mM potassium phosphate buffer, pH 7.4, containing 2% (v/v) Triton X-100. Saturated ammonium sulphate (133µL), was then added to give a final concentration of 40% (v/v). Following incubation (16h, 4°C) the samples were diluted with 40% (v/v) ammonium sulphate solution and the precipitates were filtered on Whatman GFC glass fibre filter discs (2.5cm diameter) and washed (3 x 1 ml) with the same solution by vacuum filtration on a Millipore filter apparatus. The filters were then counted for radioactivity.

2.1.b Polyethyleneimine-treated GFC filtration method

Samples, as above, were incubated (90 min, 23°C) and then filtered on GFC filters which had been presoaked in 0.3% (v/v) polyethyleneimine (PEI), pH 10 (1 in 33 dilution of 10% (v/v) stock PEI in distilled water) for 1h prior to use, as described by Bruns et al., (1983). The filter discs were then washed under vacuum with 10mM potassium phosphate buffer, pH 7.4, containing 0.1% (v/v) Triton X-100 and then counted for radioactivity.

2.2 DEAE - cellulose filtration method

Purified AChR was assayed by a method derived from Schmidt and Raftery (1973). Triplicate serial dilutions of receptor (100µL) in toxin binding assay buffer (TBA), 10mM potassium phosphate buffer, pH 7.4, containing 0.1% (w/v) BSA, 1% (v/v) Triton X-100 and 0.01% (w/v) sodium azide were incubated (90 min, 23°C, or

overnight , 4°C) with [^{125}I]-α-BGT (4.0-10 nM, 50µL). Each sample was diluted with TBA buffer (1 ml) and applied to a double thickness of pre-moistened (with TBA buffer) DEAE-81 cellulose filter discs (2.5cm diameter). The discs were washed (3 x 1ml) with TBA buffer under vacuum and the filters were then counted for radioactivity.

Subtraction of the counts obtained in the presence of BZQ gave specifically bound radioactivity in the test sample. To ensure a sufficient excess of [^{125}I]-α-BGT, serial 2-fold dilutions of the receptor samples were made in the appropriate buffer whereby a linear relationship between dilution number and precipitated radioactivity was obtained.

The toxin binding capacity of AChR preparations is defined as the amount of [^{125}I]-α-BGT bound per ml of AChR solution (pmol/ml) by the following calculation :

$$[^{125}\text{I}]\text{-}\alpha\text{-BGT}_{\text{bound (pmol/ml)}} = \frac{\text{cpm (-BZQ) - cpm (+BZQ)}}{\text{cpm (total)}} \times \text{pmol toxin added} \times \text{sample dilution} \times 10$$

3. Purification of AChR

AChR, was extracted by using detergent from Torpedo electric organ, fetal calf and human adult skeletal muscle and was further purified from Torpedo and fetal calf by affinity chromatography. The methods used are based on those described by Stephenson et al., (1981) and Gotti et al., (1982).

3.1 Extraction procedure

Frozen tissue (200-300g) was coarsely chopped and homogenised (Waring blender, maximum speed, 1 min) in 4 vol of buffer A (10mM potassium phosphate , pH 7.4 containing 1mM EDTA, 0.1mM PMSF, 0.1mM benzethonium chloride, 2mM benzamidine hydrochloride and bacitracin; 500µg/ml). The homogenate was centrifuged (10,000g, 1h, 4°C), and the resulting pellet was homogenised, as before, in 1 vol of Buffer A containing 2% (v/v) Triton X-100 (extraction buffer) and stirred for 2-4h at 23°C or overnight at 4°C. The resulting extract was centrifuged (100,000g, 1h, 4°C) and the supernatant or 'crude' extract was filtered through glass wool.

Varying volumes of the extract were retained for use in RIA (Methods, Section B2.1) and for the estimation of toxin binding activity (Methods, Section A2).

3.2 Affinity chromatography

AChR was purified from the detergent extract by using an affinity column comprising Naja naja siamensis α-toxin coupled to Sepharose 4B.

3.2.1 Purification of α-toxin from the venom of Naja naja siamensis

α-Toxin was purified from the crude venon of Naja naja siamensis by using a modification of the method described by Cooper and Reich (1972).

Crude venom (1-2g), dissolved in distilled water (10-20ml) was applied to a phosphocellulose column (30cm x 2.5cm),

pre-equilibrated in 10mM potassium phosphate buffer, pH 6.0. By using the same buffer, the column was extensively washed to remove non-bound material, until the optical density (A280nm) returned to base level. Bound protein was eluted using a linear gradient of 10mM-500mM potassium phosphate buffer, pH 6.0, (600ml of each) at a flow rate of 70ml/h. Fractions (14ml) were collected and the optical density (A280nm) was measured.

Fractions comprising each major protein peak were pooled and solid ammonium sulphate was added to give saturated solutions (760g/L). After stirring for 16h at 4°C, the precipitates were collected by centrifugation (30,000g, 10 min, 4°C). After dissolving the precipitates in distilled water they were dialysed (16h, 4°C) against several changes of distilled water (3 x 4L). The non-dialysable material was lypholised and stored at 4°C. The protein content of each peak was determined by the method of Lowry et al., (1951).

The α-toxin content of each peak was assayed by competition with [^{125}I]-α-BGT for binding to purified Torpedo marmorata AChR. AChR (0.3pmol, 100μL), in TBA buffer (Section A 2.2), was pre-incubated (30 min, 23°C) with the α-toxin test sample (20μL). After the addition of [^{125}I]-α-BGT (0.3pmol, 50μL), in the same buffer and incubation (60 min, 23°C) the amount of [^{125}I]-α-BGT bound to AChR was determined by the DEAE-cellulose filtration method (Section A 2.2). The samples were serially diluted until less than 50% inhibition of [^{125}I]-α-BGT binding was observed.

3.2.2 Preparation of the α-toxin affinity resin

The α-toxin purified from Naja naja siamensis crude venom was coupled to cyanogen bromide (CNBr) activated Sepharose 4B (March et al., 1974) by following the method of Lindstrom et al., (1981).

3.2.2a CNBr activation of Sepharose 4B

The procedure was carried out in a fume cupboard and the solutions used were maintained at 4°C throughout. Packed Sepharose 4B beads (50ml) were washed slowly with 0.1M NaCl (1L), followed by distilled water (500ml) on a scintered glass funnel under mild suction.

The beads were resuspended in distilled water (100ml) and stirred over ice with 2M sodium carbonate (100ml) to 4°C. CNBr, dissolved in acetonitrile (2g/ml) was added to the bead suspension to give a final concentration of 50mg/ml beads. After stirring the beads for 2 min at 4°C, the mixture was rapidly filtered and washed with distilled water (500ml).

3.2.2b Coupling of the α-toxin to activated Sepharose-4B

Purified α-toxin (25mg) was dissolved in ice-cold 0.2M sodium hydrogen carbonate (100ml) adjusted to pH 9.4 with 5M NaOH. The optical density (A280nm) of the resulting solution was measured before adding the α-toxin solution to the activated Sepharose 4B beads. The mixture was stirred overnight at 4°C and then rapidly filtered and the filtrate retained for the measurement of the optical density (A280nm). Thus the amount of α-toxin bound to the beads could be assessed. The beads were

subsequently washed with distilled water (400ml) resuspended in 1M glycine (200ml, pH adjusted to 9.0 using 10M NaOH) and stirred overnight at 4°C to block unreacted groups. In order to remove traces of non-covalently absorbed material the beads were finally filtered and washed sequentially with 0.1M acetate buffer, pH 4.0, containing 1M NaCl (100ml), and 0.1M borate buffer, pH 8.0, containing 1M NaCl (150ml). This washing procedure was repeated four times, after which the beads were equilibrated in 10mM potassium phosphate buffer, pH 7.4, containing 0.1% (v/v) Triton X-100, and stored at 4°C in the presence of 0.02% (w/v) sodium azide.

After use, the affinity beads were regenerated by washing with the same buffer containing additionally 0.5M NaCl (500ml), followed by this buffer without NaCl (500ml).

3.3 Adsorption of AChR from detergent extracts

Detergent extracts of AChR (usually 200-300ml) were stirred (2-4h, 23°C or overnight, 4°C) with the α-toxin Sepharose 4B beads (20ml packed volume). The beads were then washed alternately on a scintered glass funnel with 10mM potassium phosphate buffer, pH 7.4, 1mM EDTA, 0.1mM benzethonium chloride, 0.1mM PMSF, 0.02% (w/v) sodium azide and 0.1% (v/v) Triton-100 (Buffer B) containing 0.5M NaCl and Buffer B alone (3 x 350ml of each). Any residual AChR present in the washing, representing the 'non-bound' fraction was retained and assayed for toxin binding activity (Section A 2.2).

3.4 Elution of AChR from the α-toxin beads

Two methods were used to elute the AChR from the α-toxin beads.

3.4.1 Using carbamylcholine

The beads were stirred (4h, 23°C or overnight, 4°C) with 0.5M carbamylcholine in Buffer B (50ml), followed by washing with Buffer B alone (50ml). The eluate was dialysed against Buffer B (3 x 2L, 4°C) to remove most of the carbamylcholine. The AChR protein was concentrated by passage through a column (1 x 10cm) of DEAE-cellulose (DE52), pre-equilibrated in Buffer B. The column was washed, at 4°C with Buffer B (100ml) and the receptor protein was eluted with the same buffer containing 0.5M NaCl. Fractions (1-2ml) were collected and samples assayed for [^{125}I]-α-BGT binding activity (Section A 2.2). The peak fractions containing the AChR protein were pooled and dialysed against Buffer B (3 x 2L) to remove NaCl. The purified AChR preparation was finally assayed for toxin binding activity. The receptor protein was stored at 4°C in the presence of 0.02% (w/v) sodium azide.

3.4.2 Using benzoquinonium chloride

The washed affinity beads were packed into a column (2cm x 8cm) and the receptor protein eluted with 10mM BZQ in Buffer B, directly onto the DE-52 ion-exchange column. The BZQ was recirculated (overnight, 4°C), through both of the columns by using a peristaltic pump. The DE-52 column was washed free of BZQ with Buffer B (30ml) and final elution of protein was carried out as described above by using buffer B containing 0.5M NaCl.

A general scheme for AChR purification is shown in Figure 7 (page 66). Variations in the purification procedure of AChR from different tissues is shown in the Table 7 below.

Table 7

Variations in the AChR purification procedure from different sources

Source	Extraction		Affinity Chromatography	
	Time	Temp	Time	Temp
Torpedo Electric Organ	4h	23°C	4h	23°C
	overnight	4°C	overnight	4°C
Fetal Calf Muscle	4h	4°C	2h	23°C
Human Skeletal Muscle	4h	4°C	-	-
	overnight	4°C	-	-

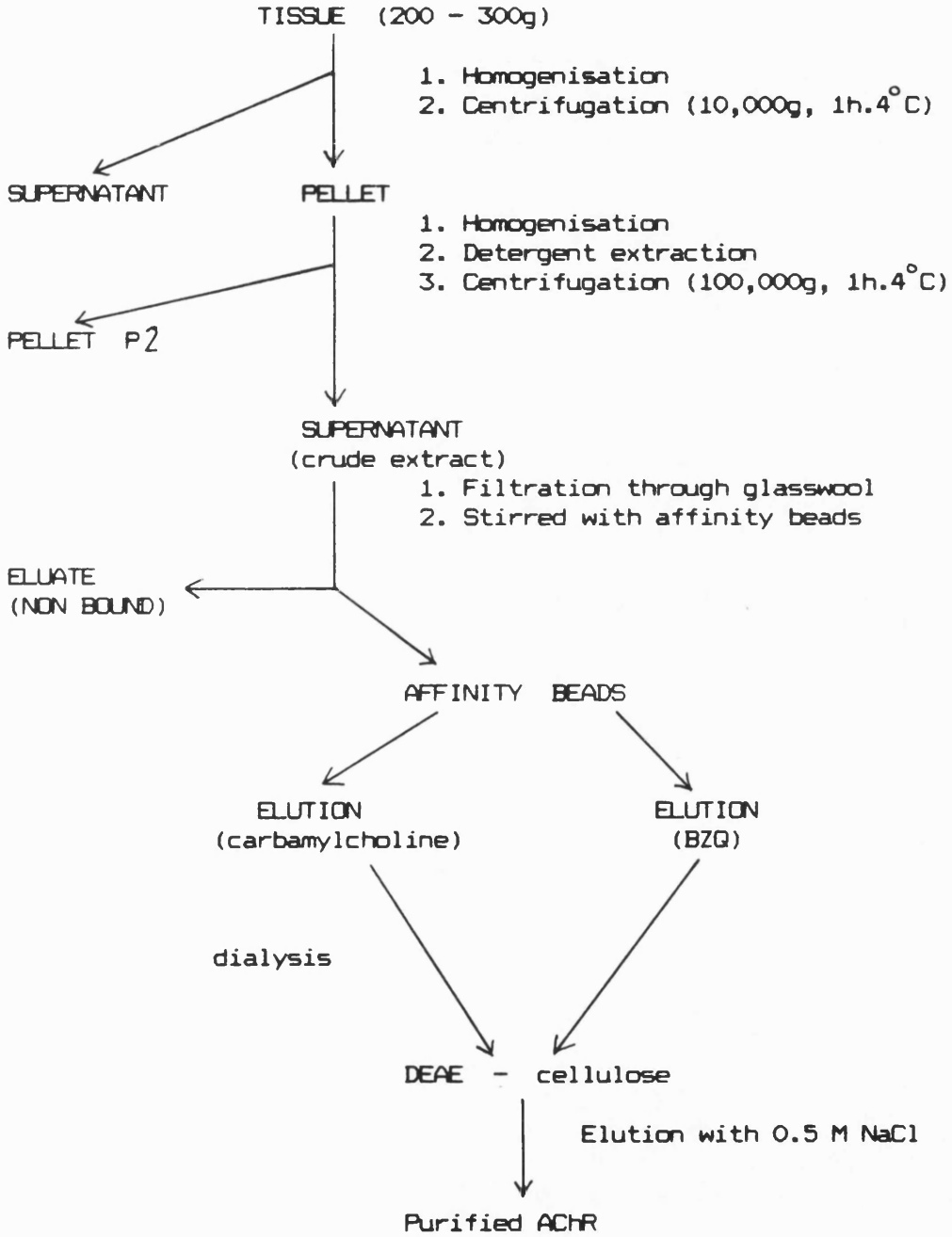
3.4 Determination of the specific activity of AChR preparations

The specific activities of AChR preparations in detergent extracts and purified samples were expressed as pmols [^{125}I]-α-BGT binding sites per μg protein.

3.5 Protein estimation

Protein was determined by the method of Lowry et al., (1951) using BSA as standard. For samples containing Triton X-100, the sodium carbonate reagent contained additionally 5% (w/v) sodium dodecyl sulphate (SDS; Wang and Smith, 1975).

Figure 7 General Scheme for AChR purification



4. Characterisation of purified AChR

4.1 SDS-PAGE

SDS-PAGE was performed according to the method of Laemmli (1970) using slab gels (16cm x 18cm). The running gel was composed of 10% (w/v) acrylamide and the stacking gel was 5% (w/v) acrylamide.

AChR samples containing 10-50 μg of protein (Lowry estimation) were boiled for 2 min with sample buffer (2% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) mercaptoethanol, 0.001% (w/v) bromophenol blue (tracking dye) in 0.625 M TRIS, pH 6.8 before loading.

The following proteins were used as molecular weight markers :

Protein	Mr
β galactosidase	120,000
phosphorylase b	94,000
IgM, μ chain	74,000
BSA	65,000
IgG, γ chain	50,000
aldolase	40,000
carbonic anhydrase	29,000

The running buffer consisted of 25mM Tris/192mM glycine buffer containing 0.1% (w/v) SDS and 10% (v/v) 2-mercaptoethanol. Samples were run into the stacking gel at 20mA and then electrophoresed at 30mA until the tracking dye was 1cm from the bottom of the slab gel.

The gel was stained for protein using 0.05% (w/v) Coomassie Blue R250 in 12.5% (w/v) trichloroacetic acid (TCA), overnight at 23°C. The gels were destained by washing, with repeated changes of 12.5% (w/v) TCA.

4.2 Gel filtration of Torpedo AChR

Purified Torpedo (7mg) was chromatographed on an ACA-34 column (2.2cm x 81cm) equilibrated in 10mM potassium phosphate buffer, pH 7.4 containing 0.1% (v/v) Triton X-100. After application of the sample, the protein was eluted from the column at a flow rate of 30ml/h and fractions (4.4ml) were collected. Samples (200µL) of each fraction were assayed for protein (Section A 3.5) and the protein peaks were pooled and assayed for α-toxin binding activity (Section A 2.2). The column was calibrated with standard proteins of known molecular weight and the fractions were assayed for protein.

RESULTS

1. Preparation of [^{125}I]-labelled α-BGT

1.1 Iodination of α-BGT

1.2 The biological activity of [^{125}I]-α-BGT

2. Assay of AChR activity

2.1 The use of PEI coated filters in the assay of [^{125}I]-α-BGT binding to detergent extracts of AChR.

3. Purification of AChR

3.1 Extraction conditions

3.2 AChR content of the tissue sources

3.3 Stability of detergent extracts of AChR

3.4 α-Toxin affinity chromatography

3.4.1 Purification of α-toxin from the venom of Naja naja siamensis
3.4.2 Preparation of the α-toxin affinity beads
3.4.3 Absorption of AChR using α-toxin affinity beads
3.4.4 Elution of AChR from the α-toxin affinity beads
3.4.5 Yield of purified AChR from Torpedo electric organ and fetal calf muscle
3.4.6 Storage and stability of purified AChR

4. Characterisation of purified AChR

4.1 SDS-PAGE analysis
4.2 Gel filtration of Torpedo AChR
4.2.1 Analysis of the protein peaks by SDS-PAGE

RESULTS

1. Preparation of [^{125}I]-labelled α-BGT

1.1 Iodination of α-BGT

α-BGT was iodinated by the chloramine T method (Methods, Section A1). A typical elution profile from G25 Sephadex column is shown in Figure 8 (page 71) and Table 8 (page 71) summarises the values obtained for the incorporation of [^{125}I] into protein and the specific activity of the [^{125}I]-α-BGT preparations.

1.2 The biological activity of [^{125}I]-α-BGT

The biological activity of [^{125}I]-α-BGT was assessed by its ability to bind to a 100 molar excess of Torpedo AChR (Methods, Section A1.2).

The biological activity of the [^{125}I]-α-BGT preparations are given in Table 8 (page 71) and is defined as :

$$\text{Biological activity} = \frac{[^{125}\text{I}]\ \text{bound}}{\text{TOTAL}\ [^{125}\text{I}]\ \text{added}} \times 100\ \%$$

2. Assay of AChR activity

Determination of the tissue content of AChR was dependant upon a suitable assay system. Ammonium sulphate precipitation of [^{125}I]-α-BGT labelled AChR is an established method for samples containing large amounts of soluble protein such as detergent extracts. The use of a simple filtration assay such as that described using PEI treated filters (Methods, Section A. 2.1.b) provided an alternative method which was examined in this work.

Figure 8 Gel filtration of $[^{125}I]$-α-BGT on Sephadex G-25.

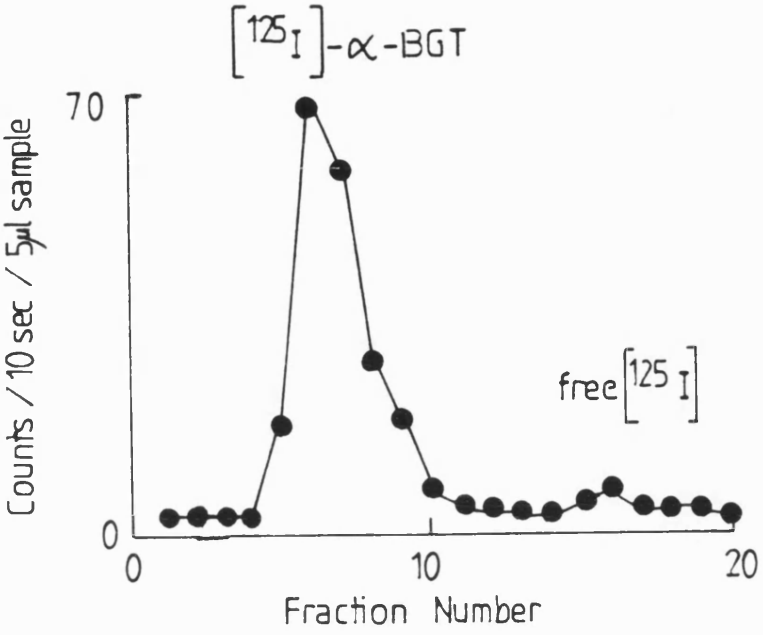


Table 8 Summary of the iodination of α-BGT

	Mean ± S.E.M.	(n)
% incorporation of $[^{125}I]$ into protein	92 ± 1.4	(12)
Specific radioactivity (Ci/mmol)	750 ± 3.4	(12)
% Biological activity	58 ± 3.2	(6)

(n is the number of determinations).

2.1 The use of PEI coated filters in the assay of [^{125}I]-α-BGT binding to detergent extracts of AChR

The non-specific retention of [^{125}I]-α-BGT on PEI treated GFC filters and untreated GFC filters in the ammonium sulphate assay in the absence of AChR (the equivalent of 'assay blanks') were compared over a range of [^{125}I]-α-BGT concentrations (Figure 9 , page74). The non-specific binding for both assay conditions was linear with increasing amounts of [^{125}I]-α-BGT and for PEI treated filters, this represented 1% of the total [^{125}I]-α-BGT added. The equivalent value for the ammonium sulphate assay using untreated GFC filters was considerably higher at 6.9%.

The quantitative aspects of the two systems for the assay of detergent extractable AChR from human and fetal calf muscle were compared (Table 9 a, below). The ammonium sulphate assay detected slightly more toxin binding activity than the PEI/GFC assay. However, the PEI/GFC assay was more reproducible, with good replicates. Also, the non-specific binding of [^{125}I]-α-BGT expressed as % of the total binding was significantly smaller using PEI/GFC filtration than in the ammonium sulphate method (Table 9 b, below).

Table 9 a Quantitative comparison of PEI/GFC filtration and ammonium sulphate precipitation in the assay of detergent solubilised AChR

	AChR measured (pmol/ml)	
Extract	PEI/GFC	Amm.SO4 (GFC)
Human Muscle	0.54	0.57
Fetal calf	0.85	0.90

Table 9 b Comparison of non-specific binding in the ammonium sulphate and PEI/GFC assay

	Non-specific binding as % of the total binding	
Extract	PEI/GFC	Amm.SO4 (GFC)
Fetal calf	32.6± 2.68	64.3 ± 2.59

Results expressed as mean ± SE. of three determinations at 1/4, 1/8 and 1/16 dilutions of fetal calf extract.

3. Purification of AChR

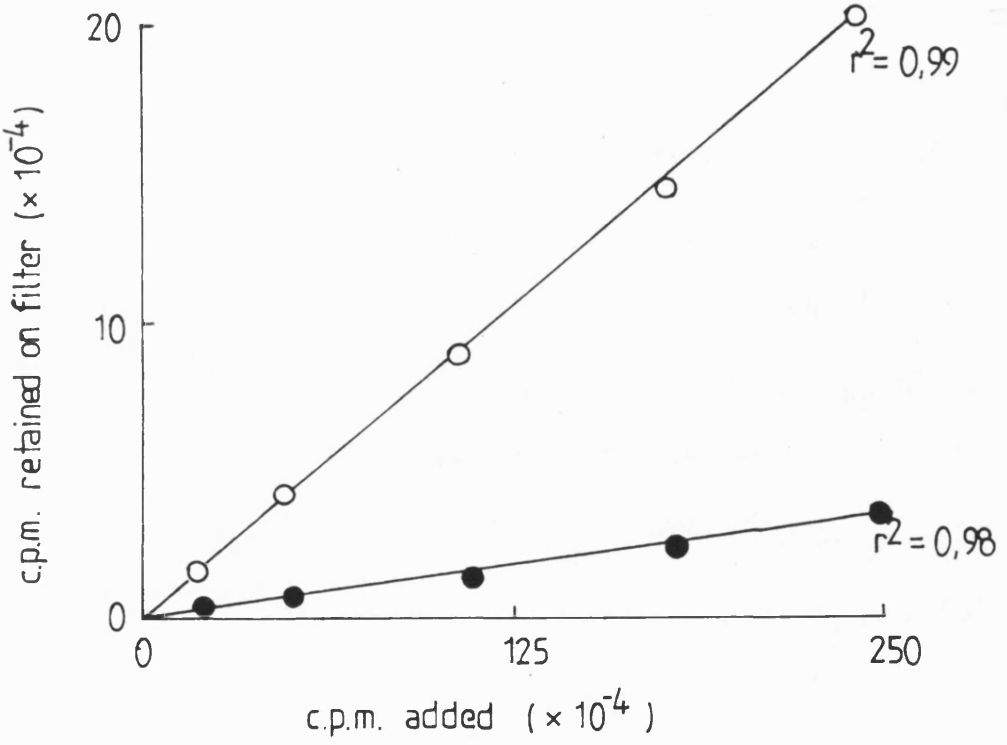
AChR was purified from two tissue sources, Torpedo electric organ and fetal calf skeletal muscle. AChR was also extracted from human adult skeletal muscle. A cocktail of protease inhibitors and anti-bacterial agents was used throughout to minimise degradation by proteolysis.

Essentially two steps were critical for the purification of receptor. Firstly, the detergent extraction of AChR from its membrane environment and secondly the use of an α-toxin affinity resin.

3.1 Extraction conditions

AChR isolated from mammalian sources is known to be susceptible to proteolytic degradation (Dolly, 1979; Einarson et al., 1982) in contrast to Torpedo AChR. In order to minimise proteolysis and obtain good yields of receptor, the time period and temperature of detergent extraction were examined.

Figure 9 Comparison of Non-specific binding to GFC filters in the PEI and ammonium sulphate assay systems



The non-specific retention of [^{125}I]-α-BGT on PEI treated GFC filters (no AmmSO4 precipitation) (-● -) and untreated GFC filters (AmmSO4 precipitation) (-○-) was compared over increasing concentrations of [^{125}I]-α-BGT (0-16nM).

For fetal calf and human skeletal muscle tissue sources the extraction conditions of 4h at 4°C and overnight at 4°C were compared. An extract sample (5ml) was removed after 4h at 4°C, centrifuged and assayed for [^{125}I]-α-BGT binding by the PEI/GFC filtration method (Methods, Section A2.1.b). This procedure was repeated after further extraction overnight. Similarly the extraction conditions of 4h at 23°C and overnight at 4°C were compared for Torpedo electric organ (Table 10 below).

Table 10 : Comparison of the efficiency of detergent extraction under different conditions of temperature and time

	TISSUE SOURCE		
	pmols/g original tissue		
EXTRACTION CONDITIONS	TORPEDO	FETAL CALF	HUMAN MUSCLE
4h at 4°C	N.D.	1.24	0.85
4h at 23°C	671	N.D.	N.D.
overnight at 4°C	682	1.30	0.98

N.D. Not determined

For fetal calf and Torpedo electric organ, additional stirring overnight resulted in increases in yield of only 5% and 2% respectively. Hence, in order to reduce proteolysis extraction for 4h at 4°C was routinely used.

Maximum yield of AChR from human skeletal muscle was obtained when additional stirring with detergent overnight was used. This resulted in an increased in yield of 14%. This could result from the more fibrous nature of the tissue source.

The conditions used for the preparation of detergent extracts of AChR from the various tissue sources are summarised in Table 7 (page65).

3.2 AChR content of the tissue sources

The AChR content from the sources used here (Torpedo electric organ, fetal calf and adult human skeletal muscle) is reflected in the amount of AChR extractable by detergent, as assessed by [^{125}I]-α-BGT binding (Methods, section A2.1) assuming good extraction. Hence, using the optimised extraction procedures described in Results, section A.3.1 the α-toxin binding per gram of starting material can be obtained (see Table 11 below). As expected, Torpedo electric organ was shown to be an extremely rich source of AChR having, approximately a 500-fold higher receptor content than human and fetal calf muscle.

Table 11 Comparison of the AChR content of tissue sources

TISSUE SOURCE	DETERGENT EXTRACT YIELD pmoles α-toxin binding sites/g ORIGINAL TISSUE
Torpedo Electric organ	850 - 1403; 948 ± 200 (n=5)
Fetal calf muscle	1.06 - 2.84; 1.95 ± 0.23 (n=15)
Adult human skeletal muscle	0.4 - 1.88; 1.13 ± 0.20 (n=10)

Results are expressed as range; mean ± S.E.M. (n)

3.3 Stability of Detergent Extracts of AChR

The stability of detergent extractable AChR was assessed by [^{125}I]-α-BGT binding at various time periods after preparation. Crude detergent extracts of human and fetal calf muscle were

very stable (Figure 10 a and b) and could be stored for up to three months without appreciable loss of toxin binding ability. The stability of detergent extracts of Torpedo electric organ were not examined.

3.4 α-Toxin affinity chromatography

Purified α-toxin was coupled to Sepharose 4B beads before being used as affinity ligand in the purification of AChR.

3.4.1 Purification of α-toxin from the venom of Naja naja siamensis

The purification of the α-toxin from crude venom was by ion-exchange chromatography on a phosphocellulose column (Methods, Section A.3.2.1). A typical elution profile is shown in Figure 11 . The α-toxin was identified by its ability to inhibit the binding of [^{125}I]-α-BGT to Torpedo AChR. Peaks A (non-bound) and F did not inhibit toxin binding to AChR, whereas peaks C and D showed the strongest inhibition. The results are summarised in Table 12 (page 80). The overall recovery of protein from the column was 65%. The fractions corresponding to peaks C and D were pooled separately and lyophilised.

3.4.2 Preparation of the α-toxin affinity beads

Lyophilised α-toxin from peak C was coupled to CNBr activated Sepharose 4B beads (Methods, Section A3.2 2b). 60-70% of the α-toxin added was consistently coupled to the resin.

The AChR binding efficiency of freshly prepared beads was tested by taking a small sample and determining its capacity to bind

Figure 10 Stability of Detergent Extractable AChR

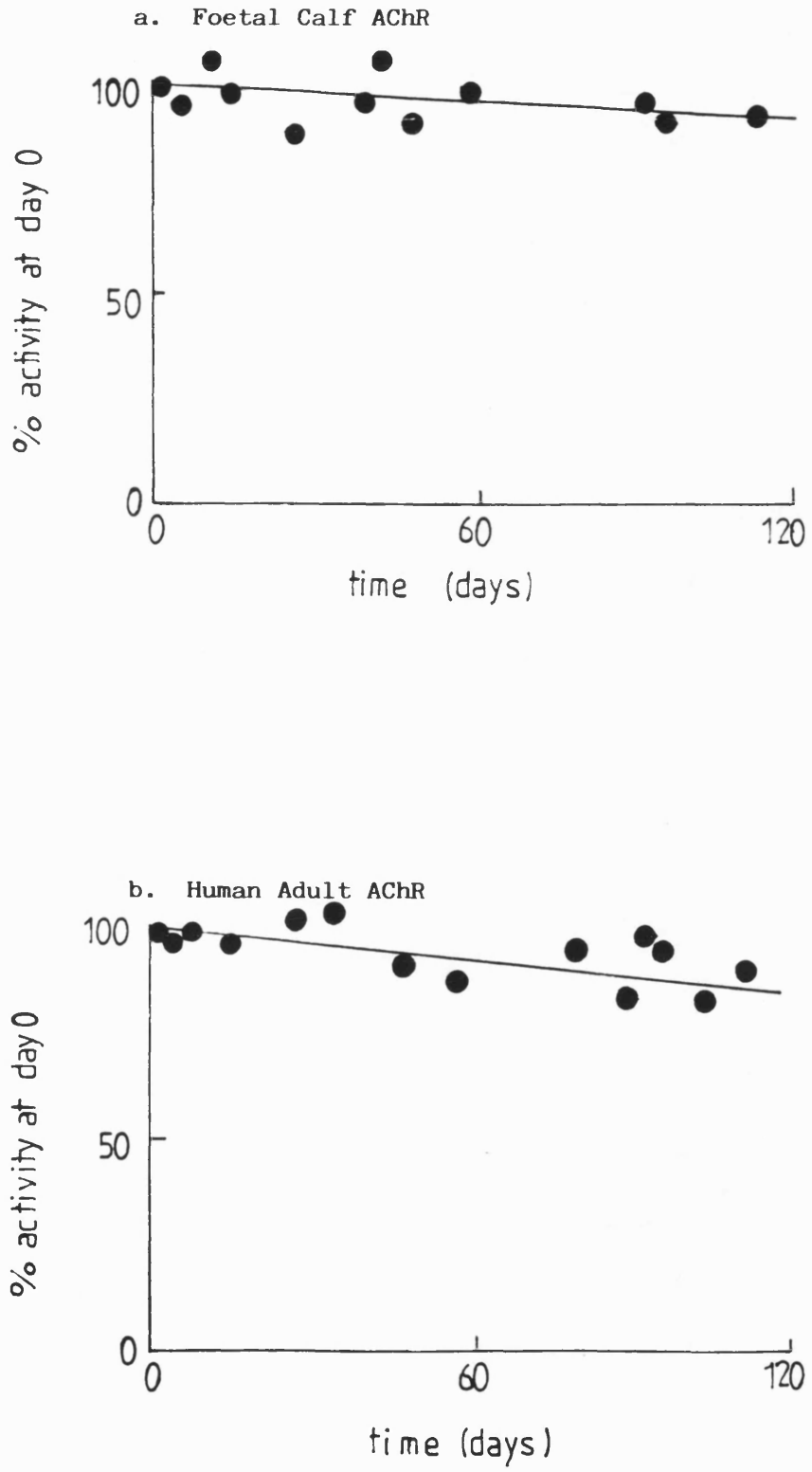


Figure 11 The elution profile from phosphocellulose ion exchange chromatography in the purification of α-toxin from Naja naja siamensis venom.

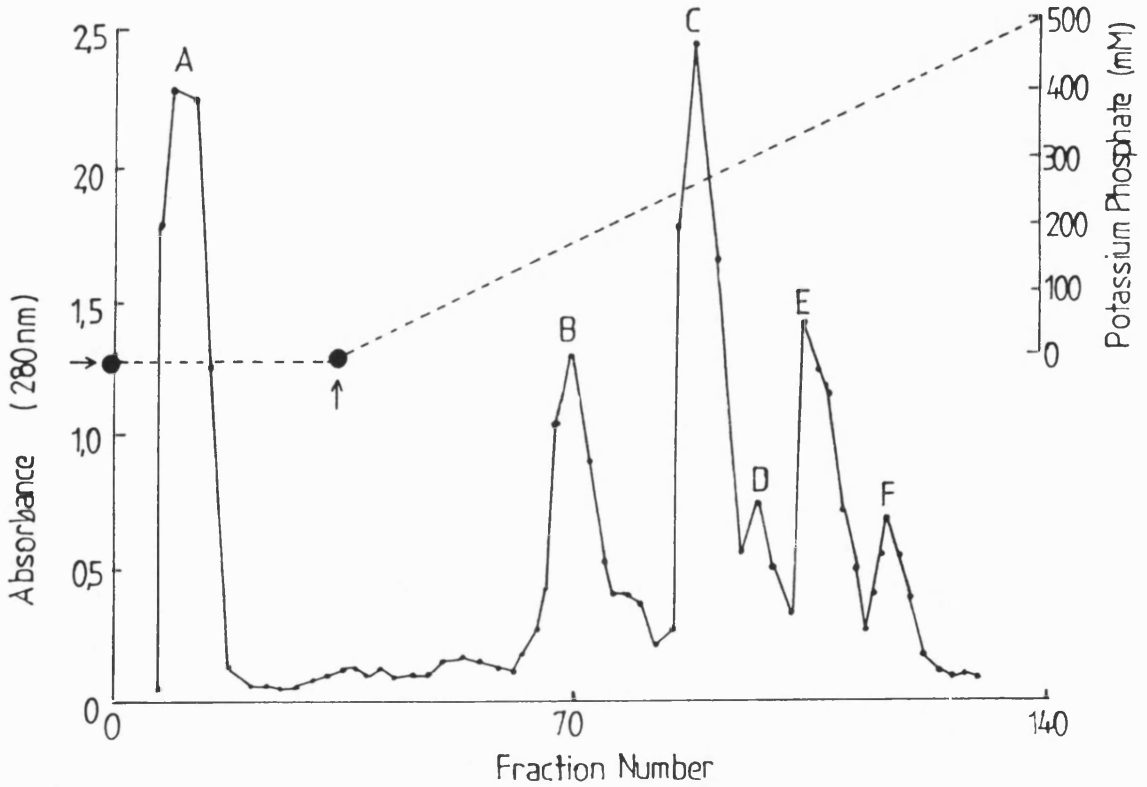


Table 12 Summary of the purification of α-neurotoxin from Naja naja siamensis

Peak	Dilution giving 50% inhibition of [^{125}I]-α-BGT binding	Total protein(mg)	pmoles α-toxin (total)	Specific activity (pmol/mg)	Purification Factor
Crude venom	1.0×10^{-5}	1750	2.32×10^{8}	1.32×10^{5}	-
A (non-bound)	No inhibition	326	-	-	-
B	3.89×10^{-3}	131.4	9.0×10^{5}	6.8×10^{3}	x 0.05
C	1.20×10^{-5}	368.0	1.75×10^{8}	4.75×10^{5}	x 3.59
D	5.98×10^{-5}	65.6	1.43×10^{7}	2.17×10^{5}	x 1.64
E	7.89×10^{-5}	198.5	2.09×10^{7}	1.05×10^{5}	x 0.79
F	No inhibition	58.4	-	-	-

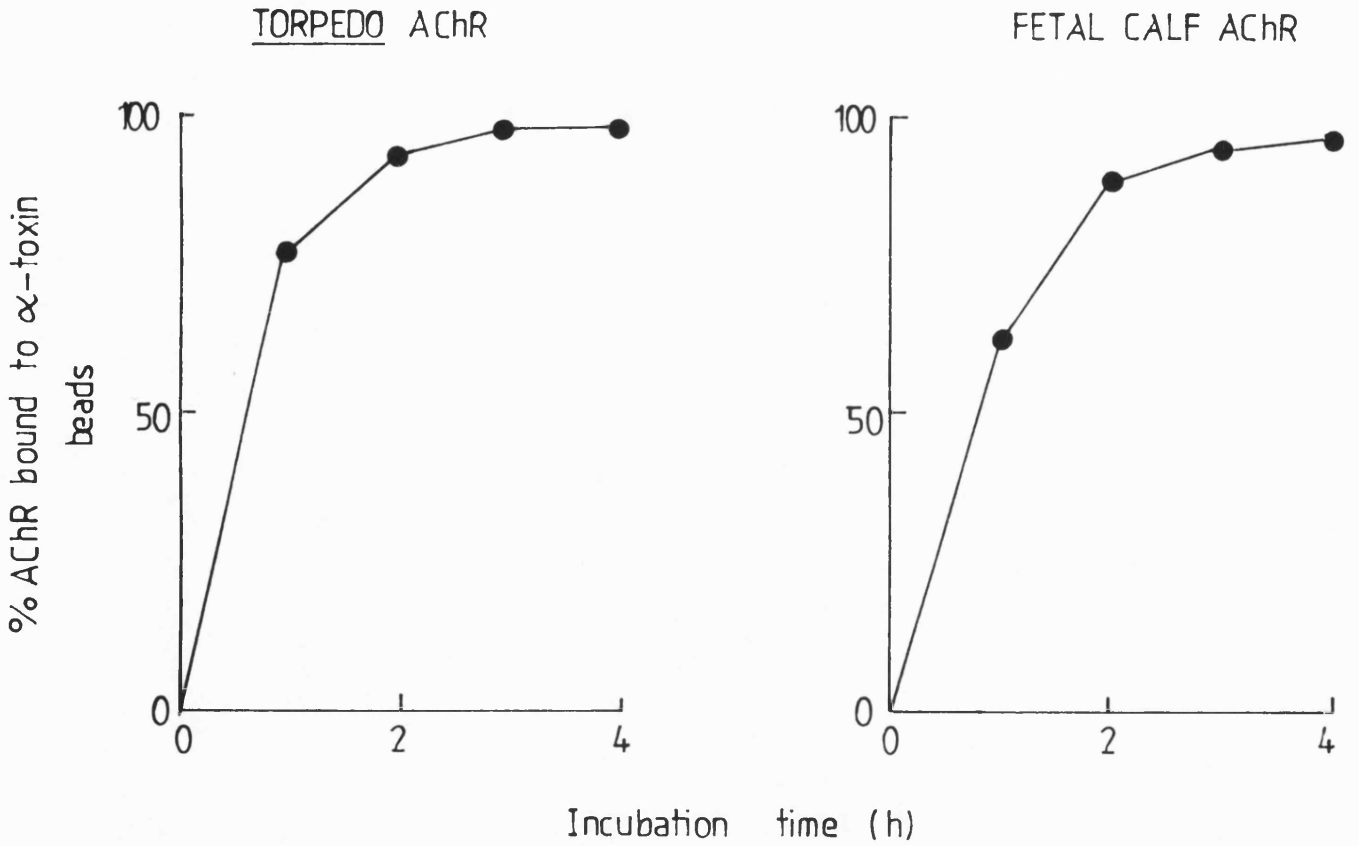
purified Torpedo AChR. The beads were usually able to bind 3.0-3.5 nmol AChR/ml of gel. There was no appreciable loss in binding efficiency over a period of one year provided that the beads were stored at 4°C and regenerated after use (Methods, Section A3.2 2b).

3.4.3 Adsorption of AChR using α-toxin affinity beads

α-Toxin - Sepharose beads were used to adsorb AChR from the detergent extracts of Torpedo electric organ and fetal calf muscle. The optimal binding of AChR from these sources to the α-toxin beads was assessed at 23°C, by removing samples of the affinity beads/extract at intervals. Following centrifugation, the supernatants, containing 'non-bound' AChR were then assayed for [^{125}I]-α-BGT binding activity (Methods, Section A2.1).

Near maximal binding of AChR occurred after 2-3h of stirring (Figure 12). To reduce the time spent in the purification of fetal calf AChR, a stirring time of 2h was chosen as 86% of the available AChR was bound by the beads. In the routine purification of fetal calf AChR, the affinity beads, under these conditions bound 84 ± 4.1% (n=7; mean ± S.E.M. (n)) of the available AChR in the detergent extract. In the preparation of purified Torpedo AChR, the affinity beads routinely bound 95 ± 1.0% (n=6; mean ± S.E.M.) of the available receptor, using stirring times of 4h at 23°C or overnight. There was no difference in the maximum binding observed using these conditions.

Figure 12 The Binding of Detergent Extractable AChR to α-Toxin Affinity Beads



3.4.4 Elution of AChR from the α-toxin affinity beads

Two cholinergic ligands, carbamylcholine or BZQ were used to elute AChR specifically from the α-toxin beads (Methods, Section A3.4). The main difference in the use of the two ligands was the necessity for an additional dialysis step when carbachol was employed, in order to remove the agonist before passage through the DE-52 ion-exchange column. With BZQ, AChR could be eluted from the α-toxin beads directly onto the ion-exchanger. Hence, the use of BZQ reduced the overall purification time by approximately 12h. It was also possible to re-use the BZQ solution several times for the purification of AChR.

The efficiency of the two agents for eluting AChR from the α-toxin affinity resin was compared. Purified Torpedo AChR was adsorbed onto α-toxin beads and equal volumes of beads incubated with carbachol (0.5M) or BZQ (10mM) for 90 min, then packed into columns (1 x 7cm) and the carbachol or BZQ recycled 5 times. The eluant was dialysed overnight against Buffer B (Methods, Section A3.4) and the non-dialysable material assayed by DEAE cellulose filter assay (Methods, Section A2.2). The two methods were equally efficient in eluting Torpedo AChR from the affinity resin (Table 13 below) but the use of BZQ elution was preferred because of the shorter time involved.

Table 13 Comparison of the Efficiency of Carbachol and BZQ elution

Elution Agent	% Recovery of [^{125}I]-α-BGT binding activity from extract
Carbachol (0.5M)	16.3 ± 2.3%
BZQ (10mM)	16.0 ± 2.9%

The results are the mean ± S.E. of 2 experiments.

3.4.5 Yield of purified AChR from Torpedo electric organ and fetal calf muscle

The recovery of purified AChR can be expressed as a percentage of the detergent extractable activity as assessed by [^{125}I]-α-BGT binding, and the yield as the recovery of purified AChR per gram of starting tissue. The yields and recoveries of AChR between the tissue sources varied greatly and the results are summarised in Table 14. The results closely parallel the yields of detergent extractable AChR per gram of starting tissue (Table 10 , page 75) and again reflects the difference in receptor content of the starting tissue. This is also highlighted in the results for the degree of purification obtained in the preparation of Torpedo AChR and fetal calf AChR. Because Torpedo electric organ provides such a concentrated source of nAChR, only a low purification factor was necessary to purify it to near homogeneity. The yields and recoveries of AChR from the same tissue source also varied and this is discussed later (Discussion, Section A).

3.4.6 Storage and stability of purified AChR

Purified Torpedo AChR was stored at 4°C in the presence of sodium azide and was relatively stable with respect to α-toxin

Table 14 Summary of the purification of AChR from Torpedo electric organ and fetal calf muscle

Tissue Source	Specific Activity pmols α-toxin binding sites/μg protein	Purification Factor	Recovery of AChR as % of extract activity	Yield of Purified AChR (pmol α-toxin binding sites/g tissue)
Torpedo electric organ	2.5-5.9; 5.1 $\pm$ 0.82,(6)	20-40; 27 $\pm$ 4.5,(6)	23-36; 30 $\pm$ 3.0,(6)	174-333; 228 $\pm$ 33 (6)
Fetal calf muscle	0.3-4.2; 3.8 $\pm$ 0.34,(7)	542-2717; 1411 $\pm$ 253,(7)	10-36; 17 $\pm$ 3.0,(7)	0.2-0.5; 0.365 $\pm$ 0.04, (6)

Results are expressed as range; mean $\pm$ S.E.M., (n).

binding activity for periods up to 2 months. For example, one preparation of Torpedo AChR retained 85% of the α-toxin binding activity after a period of storage of 10 weeks. Analysis of the AChR preparation was carried out by using SDS-PAGE (Results, Section A4.1). The degradation products were removed by gel filtration (Results, Section A4.2).

Purified fetal calf AChR stored under identical conditions could be kept for up to 2 weeks without significant losses (<10%) in α-toxin binding activity. In a trial experiment purified fetal calf AChR was frozen at -20°C in the presence of 10% (v/v) glycerol. However, after a storage period of 2 weeks under conditions of freezing, 83% of the original α-toxin binding activity was lost, indicating that freezing impairs the structural integrity of the receptor.

4. Characterisation of purified AChR

The subunit composition of purified AChR from Torpedo and fetal calf AChR was studied by SDS-PAGE. Purified Torpedo AChR was also subjected to a gel filtration procedure to assess proteolytic degradation.

4.1 SDS-PAGE analysis

SDS-PAGE of purified AChR from Torpedo electric organ and fetal calf skeletal muscle was carried out in order to investigate the subunit composition of the protein, according to the procedure described in the Methods, Section A.4.1.

Estimations of subunit apparent molecular weights were made by comparing the electrophoretic mobility of the unknown species relative to mobilities of marker proteins of known molecular weight. Electrophoretic mobility was plotted against log molecular weight for standard proteins (Figure 14). A separate calibration curve was included in every experiment. The average Rf and Mr values of the standard proteins were subjected to linear regression analysis and gave a good linear relationship with $r^2 = 0.92$.

Following SDS-PAGE purified fetal calf AChR consistently showed 5 major bands of apparent molecular weight (Mr) 39, 46, 48, 52 and 57K, lower (<30K) molecular weight components were also observed.

Purified Torpedo AChR consistently showed 4 major bands of Mr 39, 47, 52 and 62K, and the typical pattern of protein bands observed following staining by Coomassie Blue is shown in Figure 17. These values agree well with published values; these being 40K, 45K, 50K and 60K (Conti-Tronconi and Raftery, 1982). Hence it was possible to assign the 39K band as the α-subunit, the 47K as the β-subunit, the 52K band as the γ-subunit and the 62K as the δ-subunit. Two additional bands were repeatedly observed corresponding to Mr 43K and 66K.

4.2 Gel filtration of Torpedo AChR

Chromatography of purified Torpedo AChR was performed as described in Methods, Section A4.2. The receptor used had previously been stored at 4°C for 10 weeks (see Results 3.4.6)

Figure 14 Calibration of 10% polyacrylamide gel under denaturing conditions using standard proteins

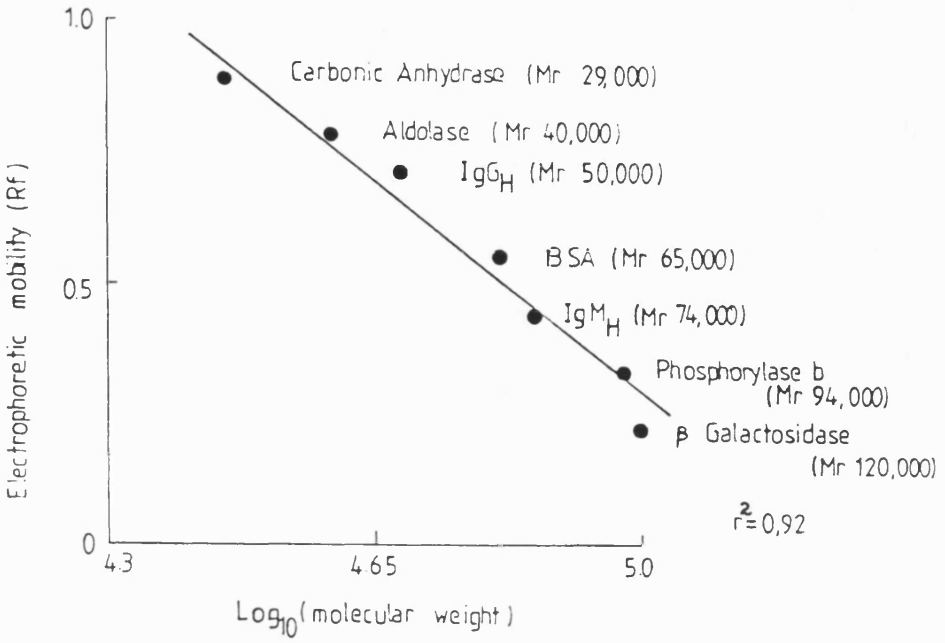
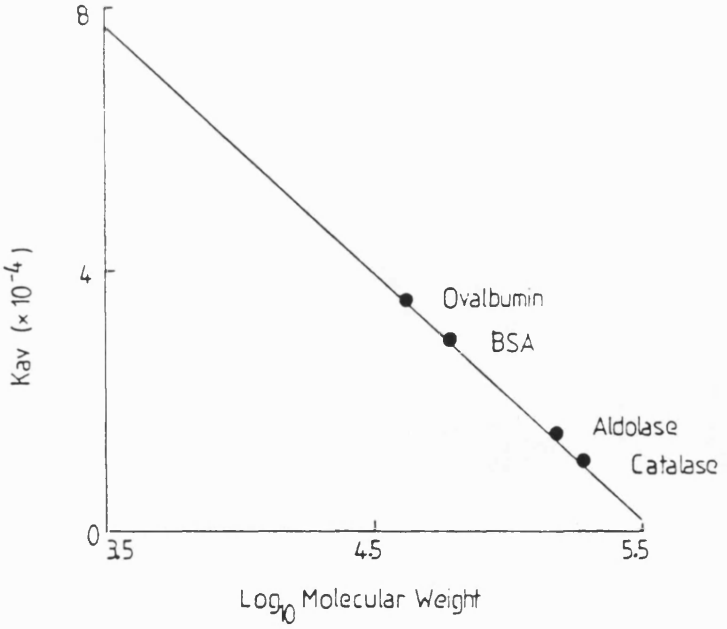


Figure 15a Chromatography of standard proteins on an ACA 34 column



The relationship between the molecular weight of the standard proteins with their Kav value where Kav represents the fraction of the stationary gel volume which is available for diffusion for that protein.

and the chromatographic procedure was used to assess proteolytic degradation.

The column was calibrated with standard proteins of known molecular weight (Figures 15a and b) by calculating Kav values, where Kav represents the fraction of the stationary gel volume which is available for the diffusion of a given solute species and can be calculated by the following equation.

$$Kav = \frac{Ve - Vo}{Vt - Vo}$$

where Ve = elution volume
Vo = void volume
Vt = total volume of the packed column bed.

Two protein peaks were observed when the Torpedo AChR preparation was chromatographed (Figure 16). These peaks represent proteins of molecular weights of 400K and 13K respectively. Following analysis of protein content and [^{125}I]-α-BGT binding activity had specific activities of 2.33 and 0.036 α-toxin binding sites/μg protein, for peak 1 and peak 2 respectively.

The specific activity of the Torpedo AChR preparation applied to the column was 1.23 pmols α-toxin binding sites/μg protein and hence the removal of small proteolytic fragments in peak 2 improved for specific activity of the preparation of AChR by a factor of 1.89. The recovery of AChR protein from the column was 84%.

4.2.1 Analysis of the protein peaks by SDS-PAGE

The protein peaks (1 and 2) obtained by the chromatography of

Figure 15b Calibration of ACA 34 column with standard proteins

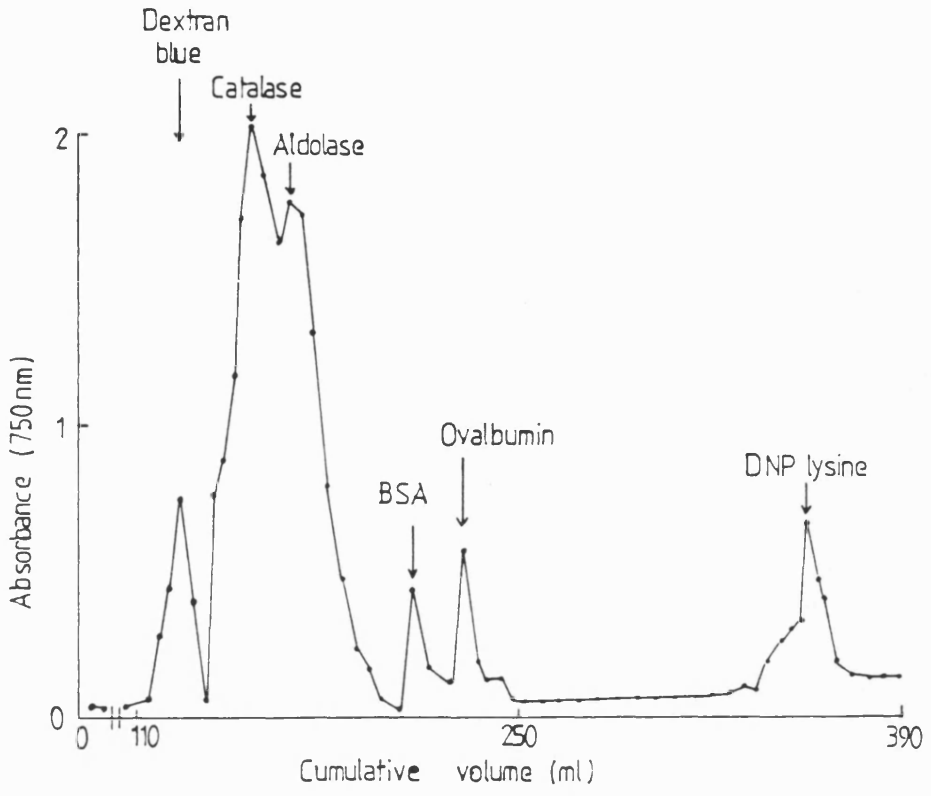
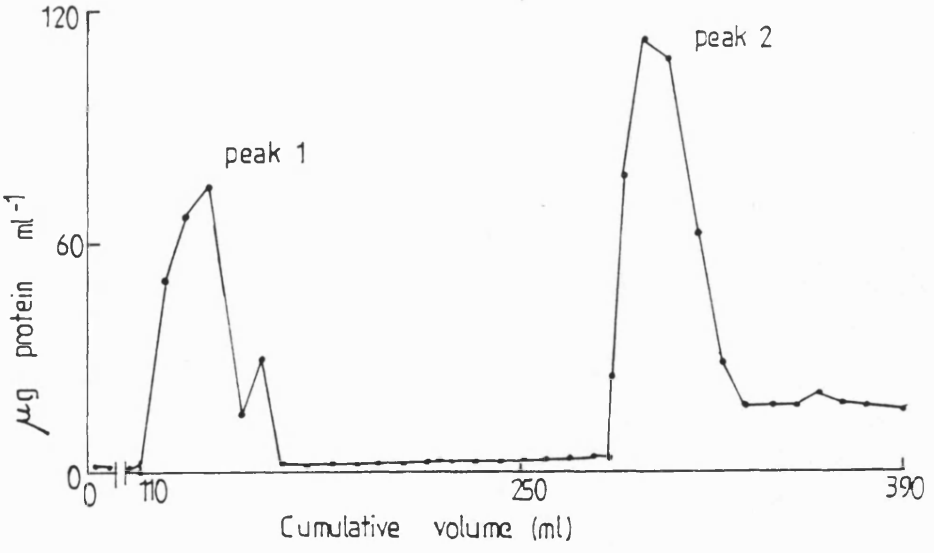


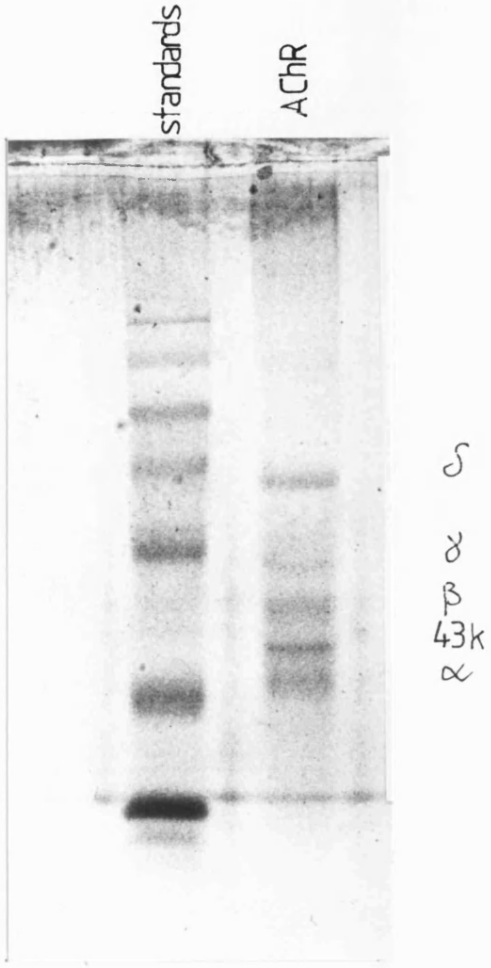
Figure 16 Chromatography of purified Torpedo AChR on ACA 34 column



Peaks 1 and 2 represent proteins of molecular weight 400K and 13K respectively.

Torpedo AChR (see previous section) were analysed by SDS-PAGE. Peak 1, appeared very similar to the original AChR preparation, showing protein bands corresponding to Mr values of 39, 47, 52 and 62K. In both peak 1 and the original unchromatographed AChR preparation a 43K band was observed but no band corresponding to a Mr of 66K was observed. Peak 2 showed no protein bands; the breakdown products being of low molecular weight (13K by gel filtration).

FIGURE 17 SDS-PAGE Analysis of AChR from *Torpedo marmorata*



SDS-PAGE analysis of AChR prepared from *Torpedo* electric organ, illustrating the four subunits α, β, γ, δ as well as the contaminating protein at 43k.

DISCUSSION

This chapter describes the purification and characterisation of nAChR protein from Torpedo electric organ and fetal calf muscle for use as immunogens in the production of polyclonal and monoclonal antibody probes (Section B). Additionally, nAChR was solubilised from adult human skeletal muscle for the characterisation of the antibodies produced (see Section B). Accordingly the purification of the nAChR from these tissue sources will be discussed here.

Advances in the isolation and characterisation of nAChR can be largely attributed to the discovery of α-neurotoxins (see Introduction, page 9), which are highly specific for the ACh binding site. Two α-neurotoxins have been used in this work; α-naja-naja toxin as an affinity ligand in the purification of nAChR and radiolabelled α-BGT for the quantitative assay of nAChR.

For a radiolabelled ligand to be of use in detecting small quantities of receptor, it must have high specific activity whilst retaining biological activity. Tritiated radiolabelled ligands are usually indistinguishable from the native ligand and have long shelf-lives. However, tritiated compounds usually have lower specific activities than those achieved by radio-iodination. Nevertheless, tritiated derivatives of α-BGT have been prepared and used in nAChR studies. (see Dolly and Barnard, 1984). However, tritium labelling procedures are complex, whereas iodination methods are relatively simple. In

addition, counting [^{125}I] is less laborious than counting [^{3}H]. Hence, iodination of α-BGT was selected for routine use.

The preparations of [^{125}I]-α-BGT had consistently high specific radioactivities with biological activities of 55-61% (Results, Section A1). Loss of biological activity may be due to damage by the powerful oxidising agent, chloramine T, used as the catalyst in the iodination procedure. Alternatively, it may result from radiation destruction, caused by the decay products of [^{125}I]. It was however accepted that the problems associated with radioiodination of α-BGT were outweighed by the high specific activities achieved, which enabled the detection of small quantities of AChR ($\sim$fmols).

Triton X-100 was shown to be an effective agent for the solubilisation of AChR from its membrane bound state (Results, Section 3.2). The use of Triton-X100 to solubilise AChR, however, has some disadvantages. The detergent absorbs strongly at 280nm and therefore necessitates the use of a modified Lowry procedure, rather than simple spectroscopic estimation of protein concentration. Triton X-100, by forming a micelle round the AChR, can also make the estimation of the molecular size of AChR difficult. However, the use of the neutral detergent Triton X-100 has several advantages over the ionic detergents sodium cholate and deoxycholate. The latter have been found to affect the ligand binding properties of the receptor, by causing a shift from high affinity states to medium and low affinity states, by allosteric interactions. (Introduction, page 6).

Triton X-100 maintains the receptor as a homeogeneous, high affinity population (Sugiyama and Changeux, 1975). Ionic detergents can also interfere with SDS-PAGE analysis and ion-exchange chromatography of the receptor protein. Hence, Triton X-100 provided the best choice for use in this study.

Although it is impossible to determine the absolute AChR content of muscle and electric organ, it is reasonable to assume that it will be reflected in the [^{125}I]-α-BGT binding capacity of the detergent extract of the tissue sources. This is, in turn, dependent upon the efficiency of extraction, the ability of all AChR present to bind [^{125}I]-α-BGT and the use of a reliable quantitative assay.

The efficiency of the detergent extraction procedure is difficult to assess because the [^{125}I]-α-BGT binding capacity of the resulting pellet (Pellet, P2; see Figure 7 , page 66) was not determined and this may be difficult due to its fibrous nature. Hence, a comparative study examining the efficiency of detergent extraction, under different conditions of temperature and time, using one tissue sample from each source was undertaken. Mammalian muscle has increased protease levels compared with Torpedo electric organ, (see Anholt et al., 1984), and extraction of AChR from fetal calf and human muscle was carried out at 4°C. Extraction of Torpedo AChR was sometimes performed at room temperature (Results, Section 3.1). Anti-protease agents were present in all buffers to limit proteolysis and the use of these agents will be discussed in a later section.

Two different assay procedures were used for the estimation of AChR in detergent extracts, both relying upon the ability of AChR to bind [^{125}I]-α-BGT (Methods, Section A.2). The use of (NH4)2 SO4 to differentially precipitate the receptor protein and the receptor-toxin complex is a well-established method (Meunier et al., 1972). However, this assay tended to give high non-specific binding and poor replicates (Results, Section A2.1). An alternative procedure used PEI-treated filters to separate free and non-bound [^{125}I]-α-BGT. The PEI imparts a net positive charge to the filter, which retains the acidic [^{125}I]-α-BGT AChR complex (pI 5.2; Conti-Tronconi and Raftery, 1982). This assay proved to be quantitatively similar to the (NH4)2 SO4 method but had greater reproducibility and lower non-specific binding (Results, Figure 9 and Table 9 a and b). Additionally, an overnight incubation step which has been reported to be critical for the (NH4)2 SO4 assay (Lotwick, 1985) could be avoided in the PEI method.

The AChR yields from Torpedo electric organ, fetal calf and human skeletal muscle varied from one preparation to another (Results, A3.2). Because of the inconvenience of obtaining fresh stocks of electric organ at frequent intervals from France (Materials), the electric organs were collected, frozen and used over a period of 22 months and variability in the observed yield of Torpedo AChR may result from deterioration over this time period.

Yields of AChR obtained from mammalian muscle were consistently lower than those obtained from Torpedo (Results, Section A3.2) and this necessitated the use of a bulk source of tissue. In this study, amputated limbs from patients with ischemic vascular disease were used as a source of human muscle. This has many advantages over the use of post-mortem tissue, because autolysis and hence proteolysis could be limited by immediate dissection of the tissue and freezing. The tissue was usually used within three weeks of freezing and the deterioration over this time was probably minimal. It is also possible that tissue stored at $-80^{\circ}C$ may lose its α-toxin binding activity during the freeze-thaw cycle. However, there was no evidence to support this. Several workers have noted variability in yields of AChR from human sources (Table 15) and this is most likely because of the inherent variability in the tissue samples used. Ischemia may result in partial denervation of the muscle and hence cause proliferation of EJR (Introduction, page 14), providing a richer source of AChR. It is however difficult to assess the AChR content of normal healthy innervated muscle for comparison. Momoi and Lennon (1982) reported very low yields of AChR (0.04-0.14 pmols/g muscle) from patients with bone cancer. However, this source cannot strictly be described as 'normal'. In a more recent study (Lotwick, 1985), muscle from a road traffic accident was used and the value quoted, 0.39 pmol [^{125}I]-α-BGT binding sites/g muscle, is at the lower end of the range of values obtained in this thesis (Results, Table 11).

Detergent extracts of fetal calf muscle gave, in general, higher yields of AChR than human muscle (Results, Table 11). However, the yields obtained in this study were generally lower than those quoted by Einarson et al., (1982) and Gotti et al., (1982) (see Table 16, page 99). In the latter studies, small fetal calves (26cm-51cm) were used, whereas this work employed a wider range of fetal calf sizes (26cm-90cm). The higher concentration of AChR in small fetal calves is probably due to the presence of EJR and also to the small size of the muscle fibres, making solubilisation easier. As the fetal calves become older, and hence larger, the muscle will become innervated and result in a decrease of AChR content. Hence the variability in yield found in this present work may reflect the differences in AChR density during muscle development. Similarly, human fetal muscle has been found to be a much richer source of AChR than adult muscle (Turnbull et al., 1985; Table 15).

Both fetal calf and adult human skeletal muscle detergent extracts were stable with respect to [^{125}I]-α-BGT binding activity (Results, Section A3.3). However, this may not reflect the true state of the receptor complex since the α-BGT binds only to the α-subunit. The β, γ and δ subunits of Torpedo amd fetal calf AChR have been shown to have greater protease sensitivities than the relatively protease-resistant α-subunit (Lindstrom et al., 1980; Einarson et al., 1982, respectively). A more precise means of assessing receptor integrity would be by immunoprecipitation wtih radiolabelled subunit specific polyclonal or monoclonal antibodies.

Table 15 Yield of AChR from fetal calf and human adult skeletal muscle

Source of Tissue	Yield (pmolα-toxin binding sites/g wet weight tissue)	Reference
Fetal calf muscle	4.1	Einarson et al., 1982
	2.5-5.3	Gotti et al., 1982
	1.6-2.84	This Thesis
Human-adult		
bone cancer	0.04-0.14	Momoi and Lennon 1982
malignant bone tumour	0.44-1.48	Lefvert, 1982
ischemic vascular disease	0.7	Stephenson et al., 1981
	0.18-2.55	Momoi and Lennon, 1982
	0.23-2.19	Lotwick, 1985
	0.66	Turnbull et al., 1985
	0.4-1.88	This Thesis
	2.7)	Vincent and
	0.9)	Newsom-Davis,
	)	1979
	)	
extraocular	0.8)	
	2.0)	
cerebral disease	0.8)	Vincent and
diabetic and peripheral neuropathy	)	Newsom-Davis,
	4)	1982
Human fetal	0.99	Turnbull et al., 1985
	0.6-1.9	Lotwick, 1985

NB: Where ranges are not quoted by the author the mean value is given.

Table 16 Purification of AChR from Torpedo and fetal calf muscle

Source of AChR	Subunit Pattern (Molecular Weight 10^{-3})	[a]Yield of Purified AChR (pmol/g tissue)	[a]Specific Activity (nmol/mg)	Reference
Torpedo AChR	40, 45, 50, 60	-	average 6.2	Conti-Tronconi & Raftery, 1982
	39, 47, 52, 62	174 - 333	average 5.1	This Thesis
Fetal calf AChR	41, 50, 53, 56	average 1.53	average 5	Einarson et al., (1982)
	42, 44, 49, 55, 58	0.30 - 0.64	4.6 - 6.8	Gotti et al., (1982)
	39, 46, 48, 52, 57	0.2 - 0.5	average 3.8	This Thesis

[a]the yield and specific activity of AChR preparations are quoted as mols ^{125}I -α-BGT binding sites/g protein.

AChR was purified from fetal calf muscle and Torpedo electric organ by affinity chromatography on immobilised α-naja naja toxin (Methods, Section A3.2). The methods used were aimed at reducing the time taken for the purification to a minimum, to limit proteolysis. Homegenisation and extraction of tissue results in the rupture of organelle membranes, ultimately leading to the release of proteolytic enzymes. Hence anti-protease agents were included in all buffers.

Early preparations of purified AChR from Torpedo electric organ and mammalian sources gave inconsistent subunit patterns (see Conti-Tronconi and Raftery, 1982, for review) and were due to proteolytic degradation of the receptor. Hence, a cocktail of protease inhibitors (EDTA, PMSF), antibiotics (bacitracin) and bacteriocides (sodium azide) was used in the extraction and purification procedures. The protease inhibitors, PMSF and EDTA, have been shown to reduce the proteolytic degradation of AChR purified from Torpedo (Lindstrom et al., 1980) and vertebrate sources (Schorr et al., 1981). Similarly the protease inhibitor, iodoacetamide, has also been shown to reduce the proteolytic degradation of the receptor (Dolly and Barnard, 1984). However, these reagents have been reported to inactivate the toxin binding activity of chick muscle receptor (Sumikawa et al., 1982a) and human receptor (Turnbull, 1984) and hence were not used in this work.

α-Naja-naja toxin coupled to Sepharose 4B was used as an affinity resin to absorb AChR from detergent extracts and was found to be a highly efficient absorbant in this present study; the resin bound 80-96% of the AChR present in crude detergent extracts (Results, Section A3.4.3). The α-toxin beads used in this work were highly substituted as defined by the criteria of Gotti et al., (1982); binding 3.0-3.5nmol AChR/ml affinity resin, a value similar to that reported by Einarson et al., (1982). The absorption of AChR from fetal calf detergent extracts to immobilised toxin (95%) was, however greater than that reported by Einarson et al., (1982) and Gotti et al., (1982), who report 67% and 49-60% absorption of AChR respectively.

Both BZQ and carbamylcholine were equally efficient in eluting receptor protein from the α-toxin affinity resin (Results, Section A3.4.4). However, the BZQ method was preferred because the procedure was faster and easier. The subsequent recoveries of AChR from Torpedo and fetal calf muscle were similar (10-36%) Results, A3.4.5) and compares well with results of other workers. Gotti and co-workers (1982) have reported that the use of low α-neurotoxin-substituted beads rather than a more highly substituted resin improves the recovery of AChR from the affinity ligand. Indeed an increase from 8% recovery to 22% was reported. These values are well within the range of recoveries found in this work using highly substituted beads and it was therefore concluded that little advantage would be gained from using a less substituted resin. The elution of AChR from the

α-toxin affinity ligand is the most costly in terms of loss of AChR, and the only partial recovery of AChR may be due to the high affinity of the α-toxin for AChR (Lee, 1979).

The variability in yields of purified AChR from Torpedo and fetal calf muscle paralleled the variability in [^{125}I]-α-BGT binding activity of their respective detergent extracts. The possible reasons behind this have been discussed previously. The yields found in this present study were within the ranges quoted by other workers (Table 16).

The degree of purification of AChR can be expressed by specific activity in terms of moles of α-neurotoxin binding sites per milligram of protein. Theoretically, the specific activity of a pure sample of Torpedo AChR should be 8nmols/mg. The values for the specific activities of purified fetal calf and Torpedo AChR found in this study (Results, Section A3.4.5) and quoted by other workers (see Table 16) fall below this ideal. Low specific activities may result from a number of factors: presence of contaminating non-receptor proteins, errors in toxin calibration and/or protein determination. Analysis by SDS-PAGE did, indeed, reveal the presence of contaminants (Results, Section 4.1) and the significance of these contaminants is discussed below. Over-estimation of the protein concentration is likely to be a significant factor in giving low specific activity values, as, in most cases, it was at the lower end of the sensitivity of the assay. Also the receptor present in a detergent micelle may behave differently from the standard

protein used in the assay. Under-estimation of the number of [^{125}I]-α-BGT binding sites in the purified receptor may have occurred due to the iodinated toxin having less than 100% biological activity (Results, Section A1.2). Additionally, the receptor is exposed to high concentrations of agonist (carbamylcholine) or antagonist (BZQ) during elution from the α-toxin affinity column and this is thought to cause desensitisation of the receptor, producing changes in its conformation and loss of ability to bind [^{125}I]-α-BGT (Changeux, 1981).

This latter phenomenon may contribute to the low (10-36%) recovery of α-toxin binding activity after absorption by the α-naja naja siamensis affinity resin.

Purified fetal calf AChR was found to be less stable with respect to [^{125}I]-α-BGT binding activity than the detergent counterpart (Results, Section A3.3 and Section A3.4.6), indicating that the purified protein is more susceptible to proteolysis. This may be due to relative concentrations of receptor in crude and purified preparations or to the concentration of Triton X-100, which could serve to protect the receptor from proteases, in the different samples. Indeed purification of the receptor may lead to increased exposure to proteases and accelerate its loss of toxin-binding activity. AChR purified from human fetal muscle is known to be highly unstable with respect to α-toxin binding ability when compared to human adult receptor (Turnbull et al., 1985).

Freezing the receptor in the presence of glycerol was detrimental to the α-toxin binding capacity of the preparation (Results, Section A3.3). This is in contrast to the findings of Lindstrom et al., (1981), but is in agreement with those of Turnbull et al., (1985). This controversy may be explained by differences in the rate of cooling of the samples. Difference between fetal calf AChR and adult AChR may reflect structural differences between the two receptor types; (J and EJR) human fetal receptor has been shown to have a lower carbohydrate content than adult AChR (Turnbull et al., 1985), and there appear to be major differences in metabolic stability between J and EJRs (see Anholt et al., 1984 for a review of differences).

In contrast, purified Torpedo AChR showed relatively stable α-toxin binding activity and this has been noted by other workers (Lindstrom et al., 1981). However, analysis of a 10 week-old preparation of Torpedo AChR by gel filtration (Results, Section A4.2) revealed the presence of small degradation products of molecular weight, 13K (Results, Section A4.2) indicating that proteolysis had occurred over this time period. The major peak comprising AChR protein was of molecular weight 400K and this value is in agreement with those quoted by other workers using gel filtration (see review by Conti-Tronconi and Raftery, 1982). The experimental evaluation of the molecular weight by gel filtration has yielded values that are consistently higher than those obtained by X-ray diffraction (250-310 KD) and sedimentation (330 KD) (Conti-Tronconi and Raftery, 1982). This discrepancy may be due to the asymmetric

shape of the AChR molecule (Introduction, page 20) which may retard the passage of the protein through the gel.

The technique of SDS-PAGE proved to be an accurate and reproducible method for identifying the four subunits of purified AChR and their molecular weights; this is highlighted by the close linear relationship between the average Rf values and average Mr values (Section A4.1; Figure 14). However, in the case of Torpedo AChR, two extra bands were seen at 66K and 43K. The 66K band was proteolysed completely on storage and this may represent acetylcholine esterase, whereas the 43KD contaminant was observed after the preparation was subjected to a gel filtration procedure (Results, Section A4.2). This may imply that this component was tightly associated with the receptor protein. Other workers have also noted the presence of 43KD proteins which appear to be distinct from the abundant cytoskeletal protein actin. Three types of 43KD protein have been reported and at least one of these has phosphatase activity. The proteins are cytoplasmic membrane components and are co-distributed with the AChR. The proteins are apparently important in stabilising the receptor in the membrane as removal by alkali ($pH \geqslant 10.5$) makes the AChR protein more sensitive to proteolysis and heat inactivation. Mabs have been documented which react with 43KD proteins and a 43KD protein can be chemically cross-linked to the β -subunit of the AChR (see Anholt et al., 1984 for a review of 43KD proteins).

SDS-PAGE analysis of purified fetal calf AChR revealed the

presence of 5 major bands of Mr 39, 46, 48, 52 and 57K. These values correspond to those reported by Einarson et al., (1982) (41, 50, 53 and 56K) and by Gotti et al., (1982) (42, 44, 49, 55 and 58K). In this latter study, the 44K protein was reported to be actin and it is likely that the 46K protein band observed in this present study was a contaminating non-receptor protein such as the proteins already discussed above or actin. This latter possibility could be examined further by using anti-actin antibodies as described by Gotti et al., (1982). Hence the protein bands observed can be assigned to the receptor subunits as follows : 39K (α), 48K (β), 52K (γ) and 57K (δ). However, further analyses by using anti-subunit specific Mabs or sequencing techniques would help verify this. Additionally, the affinity labels BAC and MBTA (see Introduction, page 19) could be used to identify precisely the α-subunit for both purified Torpedo and fetal calf AChR.

Biochemical, immunochemical and protein sequencing evidence overwhelmingly indicates that AChRs from mammalian and electric organ sources are fundamentally similar. However, there are differences in electrophoretic mobility of the respective subunits of the receptor protein from these sources as found in this study and by other workers (Table 16). This can be explained by a number of factors; experimental error; proteolysis, inherent differences in primary structure or molecular weight differences due to differences in post-translational modifications such as glycosylation and phosphorylation. The extent of glycosylation of each respective

subunit would lead to variation in molecular weight observed by SDS-PAGE. All four chains of Torpedo AChR are known to be glycosylated (Introduction 12) and there appear to be potential N-glycosylation sites present on the α, β, γ, δ and ε subunits of fetal calf AChR (Noda et al., 1985; Takai et al., 1985). However, little as yet is known about the extent of glycosylation of each subunit.

From cDNA data, Torpedo and fetal calf AChRs it is evident that the receptors from these sources have different primary structures with respect to amino acid composition and in the number of amino-acids which comprise each subunit. AChR mature subunits α, β, γ, δ comprise 437, 469, 489 and 501 amino acids (Numa et al., 1983), whilst fetal calf AChR comprises 437, 481, 497 and 561, with the ε subunit having 471 amino acids (Noda et al., 1983c; Tanabe et al., 1984; Tokai et al., 1984; Kubo et al., 1985; Takai et al., 1985). These studies thus emphasise structural heterogeneity between receptors from different sources.

SECTION B

PREPARATION OF POLYCLONAL AND MONOCLONAL ANTIBODY TO AChR

METHODS

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METHODS

1. Preparation of general immunological reagents

Several immunological reagents were used throughout this work and their preparation will be described here.

1.1 Purification of mouse IgG

IgG was purified from normal mouse serum (NMS) by affinity chromatography on immobilised Protein A (Ey et al., 1978).

1.2 Preparation of rabbit and goat anti-(mouse IgG) antisera

Normal mouse IgG, (2mg/ml, 1.0ml), previously dialysed against PBS (2L, 16h, 4°C), was emulsified with Freund's complete adjuvant (FCA; 1.0ml) and injected intramuscularly at one site in each hind leg of a rabbit or goat. The injections were repeated at 3 week intervals using IgG emulsified in Freund's incomplete adjuvant (FIA). Blood samples (10ml) were taken from the immunised animal 8 days after each injection to allow the determination of anti-(mouse IgG) antibodies. When an adequate titre was reached, the animal was bled out. The blood was allowed to clot (overnight, 4°C), then centrifuged (500g, 20 min, 4°C), and the serum collected and stored at 20°C in the presence of 0.02% (w/v) sodium azide. An adequate titre was one in which no more than 100µL of antiserum was needed to effect total precipitation of [^{125}I]-α-BGT labelled AChR-antibody complex. This complex was obtained by incubation of a fixed volume (100µL) of mouse anti-(*Torpedo* AChR) Mab supernatant, (as defined by ELISA, Section B2.2) supplemented with NMS (5µL),

with a constant volume of [^{125}I]-α-BGT labelled Torpedo AChR in a RIA procedure (Section B 2.1).

2. Detection of Anti-(AChR) antibody activity

2.1 RIA procedure

A RIA procedure similar to that described previously by Lindstrom et al., (1981) was used to detect anti-(AChR) antibodies. The method involved immunoprecipitating [^{125}I]-α-BGT- AChR - antibody complexes with an anti-globulin antiserum (Section B 1.2).

AChR (0.5nM) was incubated (45 min, 23°C) with an excess of [^{125}I]-α-BGT (5.0nM) in the presence and absence of BZQ (2.5mM). The toxin-AChR complex (100µL) was incubated (overnight, 4°C) with antisera (5µL), appropriately diluted in normal serum. The labelled AChR - antibody complex was precipitated by the addition of second antibody (Section B 1.2) and incubated for 2h at 23°C or overnight at 4°C. The precipitated complexes were separated by centrifugation (300g, 10 min, 4°C), the pellets washed (3 x 1ml) with RIA buffer (10mM potassium phosphate buffer, pH 7.4, 0.15M NaCl, 1% (v/v) Triton X-100 and 0.01% (w/v) sodium azide) and then counted for radioactivity. Assay blanks used non-immune serum. Subtraction of the counts obtained in the presence of BZQ, from counts obtained in the absence of BZQ, gave specifically bound radioactivity in the test sample. All tests were carried out in triplicate.

The antibody titre was expressed as moles of specific α-BGT binding sites precipitated per litre of serum.

For the screening of antibody production by hybridomas, (Section B 4.6), hybridoma culture supernatant (100μL) replaced immune sera. NMS (5μL) was used as a carrier protein and immunoprecipitates were formed by addition of goat/rabbit anti-(mouse IgG) antiserum (Methods B 1.2). Additional controls comprised 'spent' culture medium from X63 myelomas and an un-related Mab.

2.2 ELISA procedure

Purified Torpedo or fetal calf AChR (5μg/ml in 0.05M sodium carbonate buffer, pH 9.6 or PBS) was coated onto microtitre plates (100μL/well) by one of two methods :

a) indirectly by using microtitre plates which had previously been incubated (2h, 23°C) with poly-L-lysine (50μg/ml in PBS) or α-BGT (5μg/ml in 0.05M carbonate buffer, pH 9.6), followed by washing (3 x 10min) with washing buffer (PBS containing 0.05% (v/v) Tween-20 and 0.01% (w/v) thiomersol). AChR in PBS alone was then added.

b) directly by using AChR in PBS or carbonate buffer. In some instances, detergent extract of fetal calf AChR was used.

After incubation with AChR (2h, 37°C or overnight, 4°C), the plate was washed as described earlier. Unreacted active sites of the wells were blocked by incubation (30 min, 37°C) with washing buffer containing 1% (w/v) casein (100μL/well). After

further washing, diluted antisera (100µL) was added and incubated overnight at 4°C. Following washing, as before, the appropriate alkaline phosphatase conjugated second antibody was added (100µL; 1/500dilution in washing buffer containing 1% (w/v) casein), incubated (2h, 23°C), washed again and freshly prepared substrate solution was added (100µL p-nitrophenyl phosphate, 1mg/ml in 0.1M glycine/NaOH buffer, pH 10.4, containing 1mM MgCl2.H2O and 1mM ZnCl2). After incubation (usually 30 min, 23°C) the enzyme reaction was stopped by addition of 2M NaOH (20µL) to each well. The absorbance at 405nm was read photometrically using a Titretek Uniscan.

Normal serum and unrelated immune serum were used as negative controls. Cross-reactivity between AChR and conjugate was assessed by incubating AChR coated wells with buffer containing 1% (w/v) casein (ie: without 1st antibody) and the conjugate alone. Positive controls were immune sera defined by RIA (Methods, Section B 2.1) with established anti-(AChR) antibody activity. These controls were run with each assay.

For the screening of antibody production of hybridomas, hydridoma culture supernatant (100µL) replaced immune sera. Additional controls comprised 'spent' culture medium from X63 myelomas and an un-related Mab.

2.3 Passive haemagglutination assay

Sheep red blood cells (SRBC) coated with Torpedo AChR were used in a haemagglutination test to determine antibody titre.

2.3.1 Attachment of AChR to SRBC

Fresh SRBC were coated with AChR by the chromic chloride procedure of Ling et al., (1977). The receptor was dialysed against saline (0.14M NaCl; 2L, overnight) before use. SRBC (1ml) in Alsever's solution were washed 5 times by alternate suspension and centrifugation (200g, 10 min, 23°C) in saline. Aliquots of a 10% (v/v) suspension of SRBC (1ml) were centrifuged (300g, 10 min, 23°C) and a solution of purified Torpedo AChR (0.3mg, 300µL in saline) was added to the pellet. The suspension was mixed on a vortex; 'aged' chromic chloride (600µL, 60µg) was added dropwise and the mixture was overlaid with saline (1ml) and left overnight at 4°C. The cells were washed repeatedly, as above, until haemolysis had stopped with Hanks balanced salt solution (HBSS) comprising 0.14M NaCl, 1.46mM CaCl2, 0.81mM $MgSO_4$, 5.36mM KCl, 0.73mM KH_2PO_4, 15mM $NaHCO_3$ and 11mM glucose, pH 7.3. The SRBC pellet was finally resuspended to give a 2.5% (v/v) solution in HBSS containing additionally 5% (v/v) fetal calf serum (FCS) and stored at 4°C.

SRBC were also coated with BSA/ovalbumin in a similar manner.

2.3.2 Passive haemagglutination procedure

Serial dilutions of antiserum or hybridoma culture supernatant were prepared in 50µL volumes of HBSS containing 5% (v/v) FCS in round-bottomed microtitre trays. AChR-coated SRBC (25µL of 2.5% (v/v) was added and the end-point read by the settled pattern observed after 2-4h incubation at 23°C.

2.3.3 [^{125}I]-α-BGT binding assay on AChR coated SRBC

[^{125}I]-α-BGT (1.0pmol) was added to receptor coated SRBC (10μL of 2.5% (v/v) suspension). Non-specific binding was determined in parallel tubes by the addition of 25mM BZQ (20μL). HBSS containing 5% (v/v) FCS was added to give the final assay volume (200μL) and the mixture was incubated (90 min, 23°C). The cells were then washed (3 x 0.5ml) by using the same buffer and alternate resuspension and centrifugation (3 min, low speed, microfuge). The pellets were counted for radioactivity. Assay totals were [^{125}I]-α-BGT (1.0 pmol) alone. SRBCs coated with BSA/ovalbumin were treated similarly.

3. Production of Polyclonal anti-AChR antibodies

3.1 Anti-(fetal calf AChR) antisera

Two rabbits were immunised intramuscularly with purified fetal calf AChR (Methods, Section A3) which had been previously concentrated using an Amicon concentrator, dialysed against PBS (3 x 2L) and emulsified in an equal volume of FCA. Purified AChR (40μg/ml) was used for the first injection and subsequent injections were made at 10 day intervals by using purified protein (20μg/ml) in FIA. Animals were bled from the ear vein 2 days before the next injection, and the anti-AChR activity of the serum assessed by RIA (Section B 2.1). The rabbits were sacrificed when signs of physical distress were observed. The blood was allowed to clot (overnight, 4°C), centrifuged (500g, 20 min, 4°C), and the serum collected and stored at -20°C in the presence of 0.02% (w/v) sodium azide.

3.2 Anti-Torpedo AChR antisera

Rabbit anti-(Torpedo AChR) antisera was readily available in the laboratory.

4. Preparation of Monoclonal Antibodies

Several general procedures are common to all aspects of tissue culture work and these will be briefly described here.

4.1 General cell culture procedures

Aseptic procedure was used throughout in a sterile-air laminar flow hood (Microflow, Dent and Hellyer, Andover, Hampshire, UK). Plasticware (Nunc), all media and their supplements were obtained sterile. All other reagents were either autoclaved (120°C, 20lb/in^2, 30 min), or filter sterilised under positive pressure through sterile filter units (pore size 0.2μm diameter) before use.

All cell lines were maintained in complete culture medium consisting of RPMI 1640 culture medium supplemented with 10% (v/v) heat-inactivated (56°C, 30 min) FCS, glutamine (2mM), penicillin (100 IU/ml) and streptomycin (100μg/ml). The cells were maintained at 37°C in an atmosphere of 95% relative humidity and 5% CO_2.

Cell viability was assessed by using Trypan blue dye exclusion (2% (w/v) Trypan blue in PBS) as described by Hudson and Hay (1983). Cells were counted using a haemocytometer counting chamber (Improved Neubauer ruling).

4.2 Immunisation of mice for Mab production

Purified Torpedo and fetal calf AChR (Methods, Section A 3) were used as immunogens for the production of anti-(AChR) Mabs.

Female Balb/c mice (6 weeks old) were immunised intraperitoneally with AChR which had previously been dialysed against PBS (3 x 2L) and emulsified in FCA. Subsequent injections were made using AChR in FIA. The volume of AChR injected ranged from 0.5-1ml representing 8-35 pmols toxin binding sites (fetal calf) and usually 0.3ml Torpedo AChR representing 100-400 pmols toxin binding sites. Usually 2-3 booster injections were made 2-3 weeks apart, with a final immunisation of AChR, in PBS only, given 3-5 days prior to fusion. The serum antibody titres were monitored by RIA (Section B 2.1) to ensure that the mice were responding to the antigen.

4.3 Maintenance of X63 cell line

The hypoxanthine, aminopterin, thymidine (HAT) sensitive plasmacytoma cell lines X63.Ag8.6.5.3 (X63; Kearney et al., 1979) was used as the fusion partner for Mab production.

The X63 cell line was passaged through complete culture medium containing 8-azaguanine (2×10^{-6} M) to eliminate any revertants which were not HAT sensitive. Prior to a fusion, cells were maintained in logarithmic growth phase and had viability of greater than 90%.

4.4 Preparation of macrophage feeder layers

Macrophages were harvested from the peritoneal cavity of Balb/c mice (6 weeks old) using a method described by Hudson and Hay (1983). Cells were plated out at 10^4 macrophage feeder cells/100µL/well, and were prepared either the day before or the same day as the fusion was carried out.

4.5 Fusion procedure

Immune spleen cells from Balb/c mice were fused with X63 cells using polyethylene glycol (PEG 1500) as described by Hudson and Hay (1983).

An immunised mouse was killed and the spleen removed aseptically and placed in a petri dish containing PBS (10ml). A suspension of spleen cells, free of cell clumps, was prepared and a viable cell count was performed (Section B 4.1).

The spleen cells and myeloma cells were mixed in a ratio of 10 to 1 respectively, in a 50ml centrifuge tube, centrifuged (500g, 10 min, 23°C), washed once in serum-free medium (25ml), recentrifuged and the supernatant discarded. The cell pellet was dislodged and mixed by gentle tapping and warmed to 37°C in a water bath; subsequent steps were also carried out at this temperature. PEG solution (1ml), comprising 50% (w/v) PEG in PBS, pH 7.2, containing additionallty 10% (v/v) DMSO, was added slowly with constant shaking of the cell mixture. The cells were shaken for a further 90 sec, diluted slowly by the gradual addition of PBS (1ml) over a 1 min period and a further aliquot

(20ml) over the next 5 min. The cells were centrifuged (200g, 10 min, 23°C) and the pellet was resuspended in complete culture medium (50ml). The cell suspension was seeded over 5 x 96 well tissue culture plates (100µL/well) containing macrophage feeder layers (Section B 4.4). The following day, culture medium containing HAT was added (100µL/well) to give the requisite HAT concentration required for the selection of hybrids (hypoxanthine, 10^{-4}M; aminopterin, 4 x 10^{-7}M; thymidine, 1.6 x 10^{-5}M).

After 8 days, culture supernatant was removed (100µL/well) and replaced by the same volume of fresh culture medium containing hypoxanthine and thymidine only (HT medium) and this procedure was repeated every 3-4 days.

Screening for antibody production by ELISA (Section B 2.2) was carried out when the wells were half confluent. When positive clones were identified, they were expanded by transfer to 4 well culture dishes and cloned (Section B4.6). During this period of expansion, culture supernatant was pooled for use in the RIA (Section B 2.1) to confirm the anti-AChR specificity.

Frozen stocks were made of each uncloned positive cell line as an insurance against failure during cloning.

4.6 Cloning of hybridomas

Cloning of antibody producing hybridomas was achieved by limiting dilution. The hybridomas were plated out over macrophage feeder layers (Section B 4.4) at 5 cells/well, 1

cell/well and 1 cell/2 wells, using one culture plate for each dilution. After 1-2 weeks, colonies were visible and supernatants from those wells thought to contain one colony (by inspection of the well under the microscope) were screened for antibody activity. Positive wells were expanded as above and frozen stocks kept after each cloning. The cloning procedure was repeated three times to ensure homogeneity. Stocks of Mab supernatant were stored sterile at -20°C or at 4°C in the presence of 0.01% (w/v) thiomersol.

4.7 Cryopreservation of Cells

Healthy, actively dividing cells were centrifuged (200g, 10 min, 23°C), the supernatant removed and the cells resuspended at 10 x 10^6 cells/ml in complete culture medium and left at 4°C for 30 min. An equal volume of freezing mixture (50% (v/v) FCS containing 10% (v/v) DMSO in culture medium, 4°C) was added slowly, with shaking and samples (1ml) were transferred to 2ml freezing vials. The vials were placed in the liquid/vapour phase of nitrogen for 24h before being transferred for storage in liquid nitrogen.

4.8 Recovery of Frozen Cell Cultures

Vials were removed from liquid nitrogen storage and the contents were thawed quickly in a 37°C water bath. The vial's contents were transferred to a centrifuge tube containing complete culture medium (10ml) at 23°C, centrifuged (200g, 10 min, 23°C). The supernatant was removed and the cells were resuspended in

fresh medium at a density 10^5 cells/ml. The cells were then left to recover in 4-well culture dishes.

4.9 Large scale culture of hybridomas

After three cloning procedures, large scale culture of positive hybridomas was carried out in 175cm^2/800ml tissue culture flasks. The cells were kept in logarithmic phase growth by dilution in complete culture medium and when a sufficient cell number was achieved for the production of ascites fluid, the cells were allowed to reach the stationary phase of growth by leaving them for 24-48 hours before harvesting the cells by centrifugation (20g, 10 min, 23°C). The cells were resuspended and washed once in sterile PBS by centrifugation (200g, 10 min, 23°C) and finally resuspended in PBS at 1.5 x 10^7 cells/ml.

4.10 Production of Ascites Fluid

Prior to the innoculation with hybridoma cells, Balb/c mice were injected intraperitoneally with pristane (0.5ml, 2, 6, 10, 14 - tetramethylpentadecane). The mice were then rested for 2-8 weeks. Hybridomas (1.5 x 10^7 cells/mouse), as prepared above, were injected intraperitoneally. When ascites fluid had accumulated, as evidenced by abdominal swelling, the mice were sacrified, the abdominal cavity exposed and the ascites fluid collected using a pasteur pipette. The fluid was then centrifuged (500g, 15 min, 4°C) and stored at -20°C in the presence of 0.01% (w/v) thiomersol.

RESULTS

1. Purification of mouse IgG and the production of goat and rabbit anti-(mouse IgG) antiserum.

2. ELISA for the detection of anti-(AChR) antibodies

2.1 Choice of antigen coating buffer for the direct attachment of AChR to ELISA well.

2.2 Indirect coating of AChR by using poly-L-lysine.

2.3 Optimum coating concentration of Torpedo AChR for ELISA.

2.4 Titration of rabbit anti-(fetal calf AChR) antisera.

3. Preparation of anti-(fetal calf AChR) antisera.

4. Preparation of Monoclonal Antibodies

4.1 Purity of AChR used as antigen and immunisation of mice.

4.2 Summary of the fusions and the development of the clones obtained.

RESULTS

1. Purification of mouse IgG and the production of goat and rabbit anti-(mouse IgG) antiserum

The recovery of IgG subclasses IgG1, 2a and 2b by affinity chromatography on immobilised Protein A (Methods, Section B1.1) was 48% assuming the serum concentration of the IgG subclasses to be about 8mg/ml in a Balb/c mouse (Hudson and Hay, 1983).

The second antibody for precipitating immune complexes in the RIA was prepared by immunising rabbits or a goat with IgG prepared from NMS (Methods, Section B1.2). The resulting anti-serum was monitored for the production of antibodies by the RIA procedure (Methods, Section B2.1). Adequate titres were obtained after 4 or 5 injections of purified mouse IgG. Figure 18 shows a typical saturation curve for the precipitation of receptor-antibody complex by increasing volumes of anti-(mouse IgG) antiserum. A summary of the results obtained from each series of immunisations is shown in Table 17.

2. ELISA for the detection of anti-(AChR) antibodies

An ELISA system (Methods, Section B2.2) was used to detect anti-(AChR) antibody activity in culture supernatants, as this provided the quickest and most convenient method of screening large numbers of hybridoma culture supernatants by direct transfer from culture plates to ELISA wells.

Although this method of detecting antibody has been documented (Norcross et al., 1980; Dwyer et al., 1983; Hinman et al.,

Figure 18 Saturation curve for the precipitation of receptor-antibody complex by increasing volumes of goat-anti-(mouse IgG) antiserum.

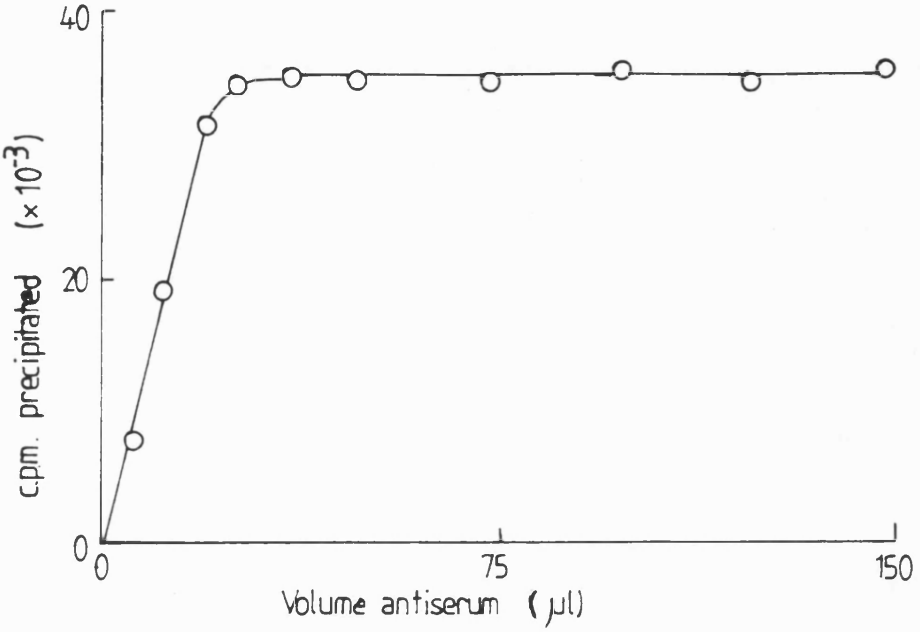


Table 17 Summary of results obtained from the preparation of goat and rabbit anti-(mouse IgG) antiserum.

Rabbit	Number of Injections	Volume of antiserum required for precipitation of AChR-Ab complex (µL)	Volume of Antiserum obtained
1	5	50	45 ml
2	4	40	40 ml
3	4	50	52 ml
GOAT-GUY	4	35	1.1L

1983; Kawanami et al., 1984; Jailkhani et al., 1986), this method had not been used previously in the laboratory for screening culture supernatants, hence a number of parameters of the assay were studied by using anti-(AChR) immune sera (Methods, Section B3).

2.1 Choice of antigen coating buffer for the direct attachment of AChR to the ELISA well

Coating the ELISA well with antigen is the initial step in this assay system. Two coating buffer systems were compared, ie: carbonate buffer, pH 9.6 and PBS, pH 7.2. The buffer systems were assessed by incubation with an excess of [^{125}I]-α-BGT and unlabelled α-BGT (see Table 18 below) to detect AChR bound to the wells. Approximately 8 x 10^5 cpm/well was added to each well.

Table Comparison of coating buffers for the direct attachment of AChR to ELISA wells

Coating Antigen	Coating Buffer	cpm [^{125}I]-α-BGT bound/well	
Torpedo AChR (5 µg/ml)	Carbonate pH 9.6	25744	528 (8)
	PBS pH 7.2	14070	139 (8)
Fetal calf AChR (5 µg/ml)	Carbonate pH 9.6	1240	35 (5)
	PBS pH 7.2	654	65 (5)

Results are expressed as mean ± S.E. (n) where n is the number of determinations.

The carbonate buffer was more effective for coating ELISA plates with Torpedo and fetal calf AChR. This buffer was subsequently used in all ELISA experiments when antigen was used to directly coat ELISA plates. However, approximately 20 fold less α-BGT binding sites were detectable when plates were coated with fetal calf AChR than Torpedo AChR, using both buffers. This may be due to the difference in specific activity of the two receptor preparations: Torpedo AChR (4 pmols α-toxin binding sites/μg protein) and fetal calf (0.6 pmols α-toxin binding sites/μg protein). Thus, for a given amount of fetal calf receptor protein coated a smaller fraction represented AChR protein.

2.2 Indirect coating of AChR by using poly-L-lysine

Antigens may be indirectly coated onto ELISA wells by poly-L-lysine spacer arms and this method was exploited for coating Torpedo and fetal calf AChR onto ELISA plates. The binding of [^{125}I]-α-BGT, as described above, was used to assess the efficiency of this method. The results are shown below (Table 19).

Table 19 The use of poly-L-lysine in the AChR ELISA method

Coating Antigen	Coating Antigen Buffer	cpm [^{125}I]-α-BGT bound/well
Torpedo AChR (5μg/ml)	PBS, pH 7.2	40208 ± 1238 (5)
Fetal calf AChR (5μg/ml)	PBS, pH 7.2	3896 ± 498 (5)
Poly-L-lysine	-	203 ± 54 (5)

The results are expressed as mean ± S.E. (n) where n is the number of determinations.

Precoating ELISA wells with poly-L-lysine improves the efficiency of AChR binding to wells compared with the direct coating method (Results B2.1). Hence, poly-L-lysine pre-coating was used for all future assays using fetal calf AChR but was not used for anti-Torpedo AChR assays, as further studies showed that direct coating of AChR in this case provided a sensitive assay (see below). Also, this avoided the possibility of detecting antibodies which may cross-react with the coating agent.

Rabbit anti-(Torpedo AChR) antiserum (antiserum T; Methods, Section B3) was used to construct a titration curve to assess the sensitivity of the Torpedo AChR directly-coated assay for the detection of anti-(AChR) antibodies (Figure 19). The antiserum T and NRS were diluted over the range 1/50 to 1/40,000. The NRS gave negligible background readings whilst the response was linear for the anti-AChR antibody over the range 1/4000 to 1/8000. Cross-reactivity of the enzyme conjugate with wells alone or wells coated with AChR gave negligible OD readings.

The anti-AChR titre of the antiserum, as determined by RIA (Methods, Section B2.1) was 2μM. Taking the limit of detection for antibody in the ELISA as a 1/20,000 dilution, the assay can detect antibody levels of 10^{-10} M and is thus a sensitive assay system.

2.3 Optimum coating concentration of Torpedo AChR for ELISA

Purified Torpedo AChR was diluted (0.1ng - 30μg/ml) in carbonate buffer, pH 9.6 and used to coat ELISA wells. The optimum coating concentration of AChR was determined by the maximum absorbance obtained using an excess of antiserum T (1/30 dilution in PBS). NRS and rabbit anti-(BSA) at the same dilution were used to assess the non-specific binding of rabbit sera to Torpedo AChR.

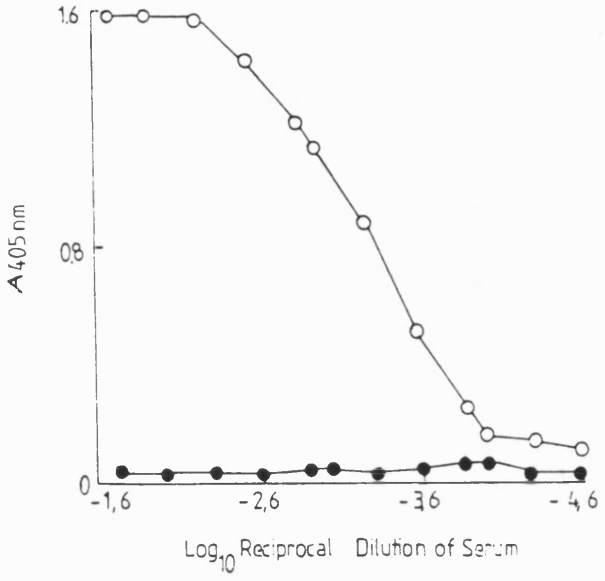
Maximum absorbance readings under fixed concentrations of conjugate, substrate and first antibody were achieved at an antigen concentration of greater than 4 μg/ml (see Figure 20). Thus, a coating concentration of 5 μg/ml was chosen for all subsequent assays; this was feasible because purified Torpedo AChR preparations have consistently high specific activities (Results, Section A.3). This coating concentration was also economical in terms of the amount of receptor used.

2.4 Titration of rabbit anti-(fetal calf AChR) antisera

Two rabbit anti(fetal calf AChR) antisera, F1 and F2 (Results, Section B3) were used to assess the ELISA system using purified fetal calf AChR as antigen on poly-L-lysine coated microtitre wells. Serial dilutions of the two antisera and NRS (1/10 - 1/5120) were used to construct the titration curves (Figure 21).

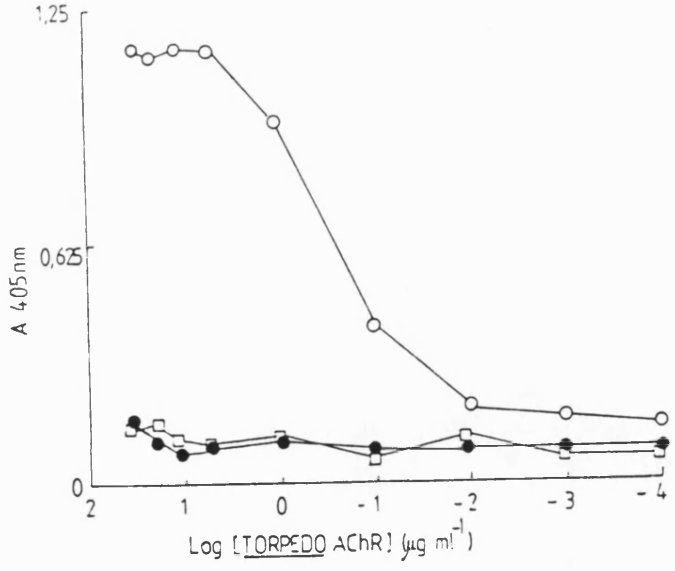
It was apparent that the amount of antigen coated onto the plates was a limiting factor as the curves were very flat at the

Figure 19 Titration of Rabbit anti-(Torpedo AChR) antiserum by ELISA



Antiserum T (-○-) and NRS (-●-) were serially diluted over the range 1/50 to 1/40,000 to construct the titration curve. Each point for figure 19 and 20 is the mean of triplicate determinations.

Figure 20 Determination of the optimum coating concentration of Torpedo AChR



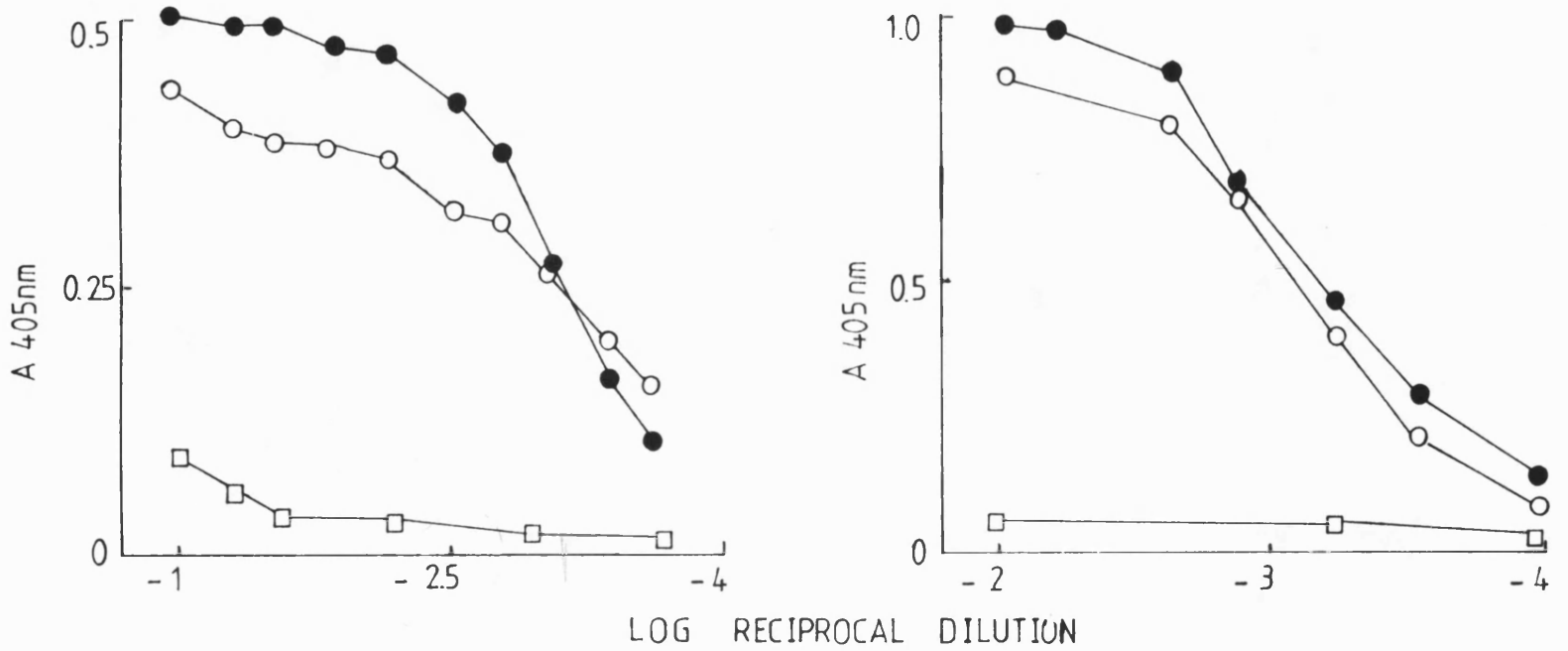
AChR (0.1ng - 30 $\mu g ml^{-1}$) was used to coat ELISA wells (Methods, Section B2.2). The optimum coating concentration of Torpedo AChR was determined by using antiserum T (1/30 dilution (-○-). NRS (-●-) and Rabbit anti-BSA (-□-) were used to assess non-specific binding.

Figure 21

Titration of rabbit anti-(fetal calf AChR) antisera on fetal calf-AChR coated indirectly by poly-L-lysine.

Figure 22

Titration of rabbit anti-(fetal calf AChR) antisera by using detergent extract of fetal calf coated indirectly by α-BGT.



Antisera F1 (-●-), F2 (-○-) and NRS (-□-) were serially diluted over the range 1/10 to 1/5120 (Figure 21) and 1/100 to 1/12600 (Figure 22). Each point is the mean of determinations made in triplicate.

higher concentrations of antibody and the optical densities obtained at these concentrations were low. It was not feasible to increase the amount of purified fetal calf AChR coated onto the wells due to the limited amount of protein available . Hence, an alternative assay system using plates pre-coated with α-BGT, followed by incubation with fetal calf detergent extract was examined. An α-BGT coating concentration of 5 μg/ml was chosen for this assay system, since this had proved effective in the assay procedures described by Hinman et al., (1983) and Jailkhani et al., (1986).

The titration curves of antisera F1 and F2 obtained using this method are shown in Figure 22. This assay system was approximately ten times more sensitive than using poly-L-lysine and purified fetal calf (Figure 21), with a good range of optical density over the antiserum dilution and neglible backgrounds using NRS.

This system was used for the screening of anti-(fetal calf AChR) secreting hybridomas.

3. Preparation of anti-(fetal calf AChR) antisera

Purified fetal calf AChR was effective as immunogen in the two rabbits examined (Methods, Section B1.2). The development of anti-(fetal calf AChR) titres, as determined by RIA (Methods, Section B2.1) using [^{125}I]-α-BGT labelled fetal calf AChR is shown in Table 20 (below) after injection of purified AChR at day 0, 10, 20 and 30. Pre-immune sera from both the rabbits had no detectable anti-(fetal calf AChR) titre.

Table Development of anti-(fetal calf AChR) antibody titres in rabbits

Days after first immunisation	10^{-9} x Anti-(AChR) antibody titre (M) Rabbit 1 ANTISERUM F1	Rabbit 2 ANTISERUM F2
8	60	187
18	795	656
28	1095	1406
37	not determined	700
38	320	Rabbit killed
	Rabbit killed	

After 3 injections of AChR, both rabbits developed signs of physical distress, ie: floppy ears and muscular paralysis of the hind limbs, and the rabbits were sacrificed. Maximum titres occurred just before the onset of physical distress and the titre fell just before the rabbits were killed.

4. Preparation of Monoclonal Antibodies

4.1 Purity of AChR used as antigen and immunisation of mice

Purified Torpedo amd fetal calf AChR (Methods, Section A3) with specific activities of approximately 3.5 and 0.6 pmols of α -BGT binding sites/μg of protein respectively were used as immunogens in Balb/c mice (Methods, Section B4.2). The purity of the preparations were analysed by SDS-PAGE (Results, Section A4.1).

The antibody response in the immunised animals was monitored by a RIA procedure (Methods, Section B2.1) at various time intervals. The results are shown in Table 21. To varying

Table 21 Antibody Titre of mouse serum after immunisation with purified AChR

	Injection Day	AChR (pmols) Injected	Day of Test Bleed	Titre x 10^{-10} M
Mouse 1	0	100	0	0.9
	30	100	NOT DETERMINED	
	65	300	68	2630
		Mouse used for Fusion T1, day 68		
Mouse 2	0	100	0	1.2
	21	150	25	600
	78	400	81	5370
		Mouse used for Fusion T2, day 81		
Mouse 3	0	12	0	1.2
	18	16	20	25
	33	20	38	85
	39	30	42	127
		Mouse used for Fusion F1, day 42		
Mouse 4	0	8	0	0.9
	15	18	19	12
	38	20	42	59
	45	25	48	87
		Mouse used for Fusion F2, day 48		
Mouse 5	0	15	NOT DETERMINED	
	15	15	19	286
	34	30	38	478
		Mouse used for Fusion F3, day 38		

Mice 1 and 2 were immunised with Torpedo AChR, mice 3, 4 and 5 were immunised with fetal calf AChR.
All titres were determined by RIA (Methods, Section 2.1).

degrees, all the mice produced antibody titres against AChR but throughout the immunisation period none showed signs of physical weakness such as muscle weakness, characteristic of EAMG. Higher titres were obtained when mice were immunised with Torpedo AChR than fetal calf AChR. This may be due to the higher specific activity of the Torpedo AChR preparations and hence the larger immunising doses.

4.2 Summary of fusions and the development of the clones obtained

Hybrid clones were produced from all fusions carried out (Table 22) with an average of 36% (n = 5) of the wells seeded showing growth. However, of these fewer wells contained clones producing antibodies towards AChR.

The fusions using fetal calf AChR as immunogen were less successful than those employing Torpedo AChR. Two positive clones were obtained but these proved to be unstable with respect to antibody production and lost activity on expansion and cloning (Table 22). Two clones resulting from the Torpedo AChR fusion showed similar characteristics, however five stable hybrids (clones B11, C11, E8, C7 and E11) were obtained and were cloned 3 times without loss of antibody production (Table 22). These hybrids were grown as ascites in Balb/c mice and subsequently characterised. Several of the mice showed signs of physical distress but it was difficult to determine whether any weakness observed was due to EAMG or to the trauma of ascites production. These Mab will be referred to as Mabs B11, C11, E8, C7 and E11.

Table 22 Summary of the fusions carried out and the fate and development of the clones obtained.

Fusion No	No of Wells seeded	No of Wells positive for growth	No of Wells positive for anti-(AChR) Ab	Colony	Cloning	Ascites produced
T1	480	220	4	B11	x 3	YES
				C11	x 3	YES
				E8	x 3	YES
				E11	Lost activity on cloning	-
T2	384	109	3	B6	Lost activity on cloning	-
				C7	x 3	YES
				E11	x 3	YES
F1	480	107	0	-	-	-
F2	400	174	1	H5	Lost activity on expansion and cloning	-
F3	480	188	1	B3	Lost activity on expansion and cloning	-

Fusions T1 and T2 employed Torpedo AChR as the immunogen.
" F1, F2 and F3 employed fetal calf AChR as the immunogen.

DISCUSSION

EAMG has been produced in a number of animals by injecting AChR purified from a number of species (see Lindstrom, 1979 for review). The disease was first described in rabbits by Patrick and Lindstrom, (1973) using purified eel receptor and indeed the majority of studies have used electric fish AChR to induce EAMG. There are, however, few reports of the induction of EAMG by using mammalian AChR (see Harrison and Behan, 1986) and only one other report, other than this present work has reported EAMG induced in rabbits using fetal calf AChR (Lindstrom, 1979). In this present work maximum anti-(fetal calf AChR) titres occurred just before the onset of EAMG, after which the titre fell sharply (Results, Section B3) and this correlates well with the findings of other workers using similar amounts of electric fish AChR as immunogen (Lindstrom, 1979).

EAMG in this present study was assessed by observation only, and this could have been further verified by studying electromyographic responses to repeated nerve stimulation. Alternatively the effects of an anti-acetylcholinesterase drug, such as neostigmine could have been used to see if the drug could eleviate the symptoms of the disease. Unlike the rat model of EAMG there appears to be no distinction in rabbits between the early acute and later chronic phases of the disease and indeed anti-immunogen antibody titres have been reported to show a general correlation with disease severity in rabbits (see Harrison and Behan, 1986). In general, the AChR content of

muscles from the immunised animal is reduced due to the pathological activity of the antibodies (see Introduction, page 38) and this activity is dependent upon the level of anti-(rabbit AChR) antibodies and the extent of cross-reactivity of host receptor with rabbit anti-(fetal calf) AChR antibodies. Hence it would have been interesting to monitor these factors during the course of the immunisation program. Similarly the levels of anti-α-BGT binding site antibodies which could interfere directly with neuromuscular transmission would have provided a useful adjunct to these studies as anti-'site' antibodies have been reported in rabbit anti-Torpedo AChR antisera by other workers (see Harrison and Behan, 1986). Although these studies were not carried out, partial characterisation of the two anti-(fetal calf antisera) F1 and F2 is given in Results, section C2 and discussed in Section C.

Screening culture supernatants for antibody requires a reliable and rapid assay procedure and in this present work an ELISA system was developed for this purpose. The assay proved to be a valuable asset for screening hybridomas and re-clones, as a large number of assays could be performed quickly and accurately in a short period of time. The ELISA system has several advantages over the RIA procedure under these circumstances, as the RIA procedure relies on a constant supply of iodinated-α-BGT, with good biological activity whereas the commercial ELISA reagents are of a relatively uniform standard and stability. Additionally there is no need in the ELISA method for large quantities of second immunoprecipitating antibody or Protein A

reagents and the washing procedures are much simpler to perform than in the RIA.

The binding of antigens to plastic or poly-styrene wells relies fundamentally on electrostatic interactions, hence the choice of coating buffer may have an effect on the efficiency of this interaction. In this present work carbonate buffer proved to be more effective than PBS in coating plates with purified receptor (Results, Section B2.1). This finding is in agreement with reports by other workers (Norcross et al., 1980; Dwyer et al., 1983; Kawanami et al., 1984).

Under optimum conditions of coating Torpedo AChR antigen the ELISA procedure was able to detect levels of antibody comparable to the RIA and was similar in sensitivity to that quoted by other workers (Norcross et al., 1980; Hinman et al., 1983). Specificity for anti-AChR antibodies in the ELISA was demonstrated in that rabbit sera known to have a high titre against BSA did not react with AChR bound to the wells. Also, non-specific binding of NRS immunoglobulin or enzyme conjugate to the AChR or to the well itself was negligible (Results, Section B2.3).

Less success was obtained in this present study by using direct coating of fetal calf AChR than with Torpedo AChR (Results, Section B2.1) and this has been previously reported by Dwyer et al., (1983). One of the difficulties is having enough purified protein of high specific activity to coat the plates. Two additional approaches were used to increase the sensitivity of

the ELISA using fetal AChR. The first employed poly-L-lysine and purified receptor and indeed an increase in the binding of AChR was observed over the direct coating method (Results, Section B2.2), in agreement with the findings of Kobayashi et al., (1984). The polymer is a basic amino acid and presumably increases the electrostatic attraction between the negatively charged antigen to the solid phase. This indirect coating method however has been associated with increases in non-specific antibody binding (Ko bayashi et al., 1984) although this was not noted in this present study. One other potential drawback to the use of poly-L-lysine is the possibility that certain antigenic determinants of the AChR may couple preferentially to poly-L-lysine and will not, hence, be detected by this assay system.

The second method involved the indirect attachment of receptor protein from detergent extracts of fetal calf muscle by α-BGT. This resulted in an increase in sensitivity of the assay system for detecting anti-AChR antibodies (Results, Section B2.4) and similar approaches have been successfuly employed by other workers (Norcross et al., 1980; Hinman et al., 1983, Jailkhani et al., 1986; see Table 23 for a summary of ELISA systems described to date). This method has the advantage that it does not depend upon the purification of AChR, however it should be noted that antibodies directed at or near to the α-BGT binding site of AChR will not be detected using this method. Direct coating of detergent extracts of mammalian muscle has also been reported (Kobayashi et al., 1984; Jailkhani et al., 1986) this,

has however the disadvantage of the possibility of binding non-AChR proteins present in the extract to the plate, whereas the use of α-BGT specifically isolates the AChR from the extract and washing procedures ensure that the other proteins are removed. Another method, described in the literature is indirect coating by a Mab intermediate, (Dwyer et al., 1983) this system obviously depends upon the availability of a suitable Mab. The ELISA thus developed in this present study provided the basis for the detection of anti-AChR directed Mabs.

Table 23

ELISA systems described to date for the detection of anti-AChR antibodies

Details	Reference
Direct coating of purified receptor	Norcross et al., 1980 Kawanami et al., 1984 This Thesis
Direct coating of receptor extract	Kobayashi et al., 1984 Jailkhani et al., 1986
In-direct coating of purified receptor by :-	
α-BGT	Norcross et al., 1980 Hinman et al., 1983
poly-L-lysine	This Thesis
Indirect coating of receptor extract by : -	
Mab	Dwyer et al., 1983
poly-L-lysine	Kobayashi et al., 1984
α-BGT	Jailkhani et al., 1986 This Thesis

All immunogens were emulsified in FCA or FIA before being injected into animals. The production of the oil emulsion ensured that the antigen was released slowly into the animals circulation and the additional presence of bacteria in FCA helped to stimulate the animals immune response to antigen after the first injection. The immunisation of mice with Torpedo and fetal calf AChR resulted in the detection of anti-(AChR) antibodies in their sera. However, despite this antibody response, which in the case of mice immunised with Torpedo AChR resulted in very high anti-(AChR) titres (Results, Section B, Table 19) none of the mice appeared to suffer from symptoms of EAMG. Strains of both mice and rats vary in their susceptibility to this experimental disease and although the Balb/c strain has been shown to be genetically susceptible to EAMG, mice within a susceptible breed can show resistance (Fuchs et al., 1976) and this was also demonstrated in this present work.

The plasmacytoma fusion partner has been shown to rescue preferentially large recently activated B lymphocytes rather than terminally differentiated plasma cells (Samoilovich et al., 1987). Hence, for Mab production a final injection of AChR was given 2-4 days before the fusion procedure on the rationale that this should localise recently activated B lymphocytes in the spleen.

The fusion of mouse spleen cells and the myeloma cell line was carried out by using PEG. Although PEG is very toxic to cells

this agent appears to promote the close apposition of cell membranes by reducing membrane charges, while the presence of DMSO helps to modify the cell membrane and enhance fusion. By using a short exposure time (1 min) of the cells to the fusion mixture toxicity to the cells was minimised and this was reflected in the good growth of hybridoma colonies after the fusion procedure (Results, Section B, Table 22).

The number of anti-(AChR) secreting clones detected represented only a small fraction of the total number of clones screened. Of these 4 clones were unstable and lost specific antibody production during cloning. This loss of antibody producing clones may be due to overgrowth by non-antibody producing cells in the original fusion well or random chromosome loss, as cell division proceeds, which may lead to loss of chromosomes coding for antibody production. The loss of specific antibody production by anti-(AChR) secreting colonies has also been reported by Mehraban et al., (1984). Cloning by limiting dilution resulted in five stable anti-(AChR) secreting cell lines (Results, Section B, Table 22) and the properties of these Mabs is reported and discussed in the following section.

SECTION C

CHARACTERISATION OF POLYCLONAL AND MONOCLONAL ANTIBODIES TO AChR

METHODS

1. Titres and cross-reactivity of polyclonal antisera

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METHODS

The production of polyclonal and monoclonal antibodies to nAChR from fetal calf muscle and Torpedo electric organ has been described in Section B. Following this a preliminary characterisation of the antibodies was carried out. This Section summarises the methods used.

1. Titres and cross-reactivity of polyclonal antisera

The titres and cross-reactivities of the polyclonal antisera T , F1, F2 (Methods, Section B3) were determined using syngeneic and xenogeneic AChR prepared from Torpedo electric organ, fetal calf and human muscle using the procedure given in Methods, Section B.2.1.

In a comparative study the titres were also determined using the passive haemagglutination technique (Methods, Section B.2.3).

2. Antigenic characterisation of Torpedo anti-(AChR) Mabs

Several methods were used for a preliminary characterisation of the Torpedo anti-(AChR) Mabs: B11, C7, C11, E8, and E11. The titre of both the ascites fluid and culture supernatant forms of the Mab were determined, whilst the majority of the cross-reactivity studies were carried out using ascites fluid.

2.1 Titre and cross-reactivity studies of Mab ascites fluid and Mab culture supernatants

The titre and cross reactivity of anti-(AChR) Mab ascites fluid was carried out using a RIA procedure and passive haemagglutination assays as given above.

The titre of the Mabs supernatant was also studied by ELISA in addition to RIA and haemagglutination studies (Methods, Section B2). An adaptation of the ELISA technique was also used to examine the cross-reactivity of the Mab supernatant with nAChR expressed by cultured rat myotubes.

2.1.1 Determination of cross-reactivity of Mab supernatant with AChR expressed by rat myotubes by ELISA

Cell cultures of rat myotubes were prepared and after 7 days, the expression of nAChR was assessed by [^{125}I]-α- BGT binding, the cultures were then fixed and the interaction of the anti-(AChR)- Mabs with the expressed receptor was determined by ELISA. The following sections give the methods used.

(a) Culture of rat myotube monolayers

Before cell culture, tissue culture plates coated with photo-polymerised collagen gel (see Childs et al., 1985) were prepared.

Collagen solution (see Materials 1mg/ml in distilled water) was mixed with riboflavin (Flavin mononucleotide, 0.05% (w/v)) at a ratio of 4:1 (v/v). Samples of this mixture (30μl) were spread in the dark over the surface of each tissue culture well. The gel was photo-polymerised by exposure to fluorescent light in the laminar flow cabinet for 1h and dried overnight at 37^{o}C. The plates were then washed for 2-3min with distilled water and incubated with growth medium (Dulbecco's Modified Eagles Medium supplemented with 10% (v/v) Donor Horse serum, 0.15% (w/v) glucose, 100IU/ml penicillin and 100μg/ml streptomycin) for at least 30min before the addition of cells.

Myotube cultures were prepared from the thigh muscles of 1-2 day-old neonate white GFHB rats, according to the method described by Childs et al., (1985).

Rats were decapitated and the bodies placed in 70% (v/v) ethanol for 10min. Hind limbs were then removed and washed in Ca^{2+} - and Mg^{2+} - free Balanced Salt Solution (BSS).

Dissection of limbs was then carried out under a Swift binocular viewer at X10 magnification. After removal of the skin, tissue surrounding the bone was stripped and placed in a 35mm plastic dish containing BSS (1ml). The tissue was then minced with fine iridectomy scissors to produce a slurry and transferred with washing to a sterile plastic tube (final volume, 8.5ml). Deoxyribonuclease I (1mg/ml, 0.5ml) was added to prevent any cell clumping induced by deoxyribonucleic acids released from dead cells.

Crude trypsin solution (2.5% (w/v), 1ml) was added and the tissue suspension was incubated for 1h at 37°C with intermittent mixing. The cell suspension was then centrifuged (400g, 5min., 23°C) and the tissue pellet resuspended in 5ml of growth medium. Following the mechanical disruption of the tissue by the repeated passage of the tissue through a small bore glass pipette (15-20 cycles), the mixture was left to stand for 3 min and the supernatant, containing the single cell suspension was removed for filtering. Growth medium (5ml) was added to the remaining tissue and the tissue disrupted as before. The combined cell suspensions were filtered through two layers of

nylon bolting cloth (53μm aperture) to remove remaining cell aggregates and tissue clumps. The cells were counted using Trypan Blue (Methods, Section B4.1) and added to precoated culture plate at a final density of 2.5×10^5 cells per well in growth medium (1ml). The cultures were grown at 37°C in an atmosphere of 10% carbon dioxide in air, the growth medium being replaced every 3 days. Contamination of the cultures by fibroblasts was minimised by treating 3-day-old cultures with fluorodeoxyuridine (15μg/ml) for 72h.

The cell viability of the prepared cells was greater than 90% and the yield of cells per limb was 1.27×10^6 (mean of two preparations).

Myotube cultures were routinely examined under a Zeiss inverted phase-contrast microscope before and after all assays to identify any culture wells in which the myotubes had become detached from the surface as a result of the washing procedures.

(b) [^{125}I]-α-BGT binding to rat myotube cultures

Replicate myotube cultures were washed twice with growth medium and incubated for 30min at 23°C in fresh medium with or without decamethonium (0.275ml, 1mM). [^{125}I]-α-BGT (25μl, 300nM, Methods, Section A1) was then added to all the cultures and incubated for 60min at 23°C. The cultures were then washed three times with growth medium (0.75ml, 2-3min) followed by two brief washes with PBS (0.75ml). The bound radioactivity associated with the cells was solubilised by incubation with

NaOH (0.1M, 0.3ml) for 30min at 23°C. The contents of each culture well were then transferred to LP3 tubes. Each culture well was then further incubated with NaOH (0.1M, 0.2ml) for 15min at 23°C and the washings transferred to LP3 tubes for counting.

Specific [^{125}I]-α-BGT binding to the cultures was calculated from :

$$\frac{\text{total cpm (in the absence of decamethonium)} - \text{non-specific cpm (in the presence of decamethonium)}}{\text{Specific Radioactivity (cpm/pmol [}^{125}\text{I]-}\alpha\text{-BGT)}}$$

(c) Cross-reactivity study by ELISA

On day 7 rat myotube cultures were fixed and maintained at 4°C until use in 0.04% (w/v) paraformaldehyde (diluted from 4% (w/v) stock paraformaldehyde pH 7.4, containing additionally 90mM sodium phosphate buffer and 0.02% (w/v) NaCl).

For cross-reactivity studies with anti-(Torpedo) AChR Mabs, the fixative was removed and the cultures washed with PBS (3 x 1ml). PBS containing 5% (v/v) NRS was then added (1.0ml) and left for 1h to block non-specific binding. After removal of 0.75ml of blocking agent, anti-(Torpedo AChR) and control Mab supernatant and normal culture medium (250µL) was added to the culture wells. Each determination being made in triplicate. After incubation (2h, 23°C), the supernatants and culture medium were removed and the cultures washed (3 x 1ml) with PBS containing 5% (v/v) NRS. Horseradish peroxidase conjugated goat anti-mouse

IgG(0.5ml, 1/500 dilution in PBS/5% (v/v) NRS) was added and incubated (1h, 23°C) and the cultures then rinsed in PBS (3 x 1ml) alone. The binding of cross-reacting antibody was then detected by the addition of substrate solution (0.5ml) consisting of 0.5M sodium acetate, pH 6.0, 0.1% (w/v) 3, 3', 5, 5' - tetramethylbenzidine (TMB) and 0.005% (v/v) H_2O_2. After 30min the reaction was stopped by the removal of 100µL of reaction mixture to microtitre wells containing H_2SO_4 (25µL, 2M). Separate assay blanks consisted of culture wells treated as above but with the omission of conjugate and another with substrate. The absorption at 450nm was measured by using the mixture substrate solution (100µL) and H_2SO_4 (25µL) alone as a back-off reading.

2.2 Determination of the subunit specificity of the Mabs

The nAChR is composed of four different subunits (Introduction, Section 2) and antibodies produced to nAChR may thus be directed against any of the four subunits. Hence it is possible to determine the subunit-specificity of anti-(nAChR) antibodies by first separating the subunits by SDS PAGE then transferring them onto nitrocellulose sheets for immunodetection (Western blotting).

(a) Electrophoretic transfer of AChR protein

The method of Bittner et al., (1980) was used for the electroelution of AChR protein onto nitrocellulose paper. SDS-PAGE gels were run as described in Methods, Section A4.1 . Coomassie staining was omitted and the gel was equilibrated in distilled water for 10min followed by equilibration for 30min in

transfer buffer (25mM sodium phosphate buffer pH6.5, containing 10% (v/v) methanol). A 'sandwich' of filter paper, gel, nitrocellulose and filter paper, all presoaked in transfer buffer, was assembled. This was then placed into the transfer tank (Transblot 1, BIORAD) containing transfer buffer at 4°C, such that the nitrocellulose sheet was facing the anode. Electroelution was carried out at constant voltage (12V) for 14h at 4°C.

(b) Protein staining of nitrocellulose sheets

The strips of nitrocellulose containing electrotransferred reference proteins and AChR proteins were stained using India ink as described by Hancock and Tsang, (1983).

The strips were equilbrated in PBS containing 0.1% (v/v) Tween and 0.1% (w/v) thiomersol for 1h at 37°C prior to the addition of a solution of India ink (1µl/ml PBS/Tween buffer, as above). After the staining procedure (1h, 23°C, with constant shaking), the nitrocellulose strips were destained using distilled water.

(c) Immunobinding of anti-(AChR) antibodies

For these studies the nitrocellulose strips containing AChR protein were preincubated with PBS containing 1% (w/v) casein. This helped to reduce the non-specific binding of the peroxidase conjugated second antibody to the nitrocellulose paper.

The strips were incubated (overnight, 4°C) with appropriately diluted test antibody. All dilutions were made in PBS containing 1% (w/v) casein and suitable working dilutions were 1/5 for monoclonal culture supernatants and 1/100 for antisera

in a final incubation volume of 4mls. The strips were then washed with PBS/casein (3 x 10min, 4°C) and incubated with the relevant horseradish peroxidase conjugated second antibody, (diluted 1/1000 in PBS/casein) for 2h at 4°C. The strips were then washed as before and then finally washed with PBS alone. The binding of cross-reacting antibodies was visualised by incubation (10-15min, 23°C) with freshly prepared substrate solution (0.02% (w/v) 3-amino-9-ethylcarbazole in 50mM sodium acetate buffer, pH 5.0, 0.03% (v/v) hydrogen peroxide). The reaction was stopped by washing with distilled water.

2.3 Determination of the Mab immunoglobulin subclass

The method used is essentially similar to that described for Western blotting (Methods, Section C2.2) and here only the minor differences will be given.

Standard anti-immunoglobulins (IgG1, G2a, G2b, G3 and M) were spotted onto nitrocellulose strips, pre-washed in 50mM Tris buffer, pH 7.4, containing 200mM NaCl. Dilutions of monoclonal antibody supernatant (diluted 1/10) and NMS (diluted 1/100) were made in the above buffer containing 1% (w/v) casein. The conjugate used was horseradish peroxidase conjugated rabbit -anti (mouse IgG) (diluted 1/1000) and the stain used was 3-amino-9-ethylcarbazole.

Spent culture medium from X63 myelomas was used as a negative control, with a Mab AJ3 of known immunoglobulin subclass a positive control.

2.4 Determination of Mab affinity

The affinity of each Mab for Torpedo AChR was determined by varying the concentration of [^{125}I]-α-BGT labelled AChR using the method described by Vincent and Newsom-Davis, (1982). A constant amount of Mab supernatant (100μl) was incubated (overnight, 4°C) in a final volume of 550μl with varying amount of [^{125}I]-α-BGT-labelled AChR (0.05 - 0.45 pmols), followed by immunoprecipitation by second antibody and washing as previously described for the RIA procedure (Methods, Section B2.1). Non-specific counts were determined in parallel tubes using AChR labelled in the presence of excess BZQ. These values were subtracted from the total counts to obtain specific counts. The results were analysed by Scatchard plots (Scatchard et al., 1949).

2.5 The interaction of Mab with the cholinergic binding site of AChR by inhibition and dissociation assays

The possible interaction of the anti-(AChR) Mabs with the cholinergic binding site of AChR was studied using two methods:- the use of AChR coated SRBC and the use of DEAE filter assays.

2.5.1 Inhibition of [^{125}I]-α-BGT binding to AChR coated SRBC by Mabs

The toxin binding assay on receptor coated SRBC (Methods, Section B2.3.3) was modified to include a pre-incubation with Mab supernatant. Coated SRBC (20μl of 2.5% (v/v) solution) were pre-incubated with a suitable dilution of Mab supernatant (300μl) or control Mab anti-(BSA). In parallel tubes non-specific binding was determined in the presence of 25mM BZQ (40μl). BSS

containing 5% (v/v) FCS was added to a final assay volume (400μl).

Following incubation (30min, 23°C), [^{125}I]-α-BGT (0.2 pmol) was added. Following further incubation (90min, 23°C), the cells were washed as described previously, (Methods, Section B2.3.3) and counted. Assay controls contained 'spent' culture medium and assay totals were [^{125}I]-α-BGT (0.2pmol) alone.

For four preparations of Torpedo AChR-coated SRBC 0.38 ± 0.12pmol (mean ± SE) of [^{125}I]-α-BGT were bound per 10μl of receptor coated SRBC, 2.5% (v/v) suspension.

2.5.2 Effect of Mabs on the inhibition and dissociation of the [^{125}I]-α-BGT labelled AChR complex by DEAE filter assay

The inhibition of [^{125}I]-α-BGT binding to Torpedo AChR by Mabs was also studied by using DEAE filters to separate bound toxin from free toxin. The final assay conditions were as follows :- Torpedo AChR (20fmols, 100 μl) was incubated (overnight, 4°C) with increasing volumes of Mab supernatant (0-200 μl). in a final volume of 300μl: followed by the addition of a 3-fold excess of [^{125}I]-α-BGT (30fmols, 50 μl). After 2h at 23°C the incubation mixture was applied to DEAE cellulose filter discs and washed with TBA buffer and counted for radioactivity. Parallel assay tubes contained BZQ at a final concentration of 2.5mM and the results were expressed as a percentage of the counts obtained in the absence of Mab.

Mab supernatants Mab B11 and anti-(BSA) were used as controls.

The effect of Mab on the dissociation of [^{125}I]-α-BGT AChR complex was also studied using a similar method to that described by Mehraban et al., (1984).

Torpedo AChR was complexed with a two-fold excess of [^{125}I]-α-BGT in TBA buffer (Methods, Section A2.2); 2.5pmol of the complex were incubated in a final volume of 2ml in TBA buffer containing 40 μl of ascites fluid or NMS. At intervals, aliquots were removed and filtered on DEAE cellulose filters and washed extensively with TBA buffer (10ml). Specific counts were determined by pre-incubation of AChR with excess BZQ (2.5mM final concentration) before addition of [^{125}I]-α-BGT.

2.6 Investigation of the interaction of Mab with the cholinergic binding site of AChR by ELISA

The ELISA also proved a useful method for studying the interaction of Mabs with the cholinergic binding site of AChR. Two different studies were used and these are summarised below.

In parallel studies, plates were coated with Torpedo AChR only or α-BGT followed by Torpedo AChR in the usual manner (Methods, Section B2.2). The plates were then incubated with serial dilutions of Mab supernatant and the standard ELISA procedure was used thereafter (Methods, Section B2.2).

The effect of cholinergic ligands on the binding of Mabs to immobilised AChR was also studied using a method similar to that described by Watters and Maelicke, (1983). Serial dilutions of Mab culture supernatant were incubated with immobilised AChR in the absence and presence of cholinergic ligands.

ELISA wells were coated with Torpedo AChR as described in (Methods, Section B2.2). Solutions of cholinergic ligands (50 µl) in PBS containing 0.05% (v/v) Tween were added to each well except control wells (no ligand), followed by the addition and mixing of 50 µl of serially diluted (1 in 2) Mab supernatant. The mixture was incubated (overnight, 4°C) followed by the standard procedure of washing, incubation with enzyme-conjugated second antibody and development (Methods, Section B2.2). Three cholinergic ligands were used :- carbachol, d-tubocurarine and BZQ at final concentrations of 2mM, 1mM and 2.5mM respectively.

RESULTS

1. Titres, cross-reactivity and subunit-specificities of polyclonal antisera

2. Antigenic characterisation of anti-(AChR) Mabs

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2.5.1 Inhibition of [^{125}I]-α-BGT binding to AChR coated SRBC

2.5.2 Inhibition of [^{125}I]-α-BGT binding to AChR using DEAE filter assay

2.5.3 Effect of Mabs on the dissociation of the [^{125}I]-α-BGT labelled AChR complex

2.6 Investigation of the interaction of Mab with the cholinergic binding site of AChR by ELISA

2.6.1 Effect of precoating plates with α-BGT on the titration of Mabs against immobilised AChR

2.6.2 Effect of cholinergic ligands on binding of Mab to immobilised AChR

Both polyclonal and monoclonal antibodies were produced in this work with the aim of using these antibodies in the study of AChR on human lymphocytes. The objective of this section was to provide basic information on the antigenic specificities of the antibodies, and to determine whether any of the Mabs interfered with cholinergic ligand binding to AChR, in the hope that such antibodies would be of use to study ligand binding to AChR on human lymphocytes.

1. Titres, cross-reactivity and subunit specificities of polyclonal antisera

The titres and cross-reactivities of the polyclonal antisera raised against Torpedo AChR (designated T) and fetal calf-(AChR) (designated F1, F2) (Results, Section B3) were determined by RIA (Methods, Section B2.1) by using preparations of AChR from fetal calf, human muscle and Torpedo electric organ. The titres of the antisera were also assessed by a passive haemagglutination assay using SRBC coated with Torpedo AChR (Methods, Section B2.3) in a comparative study.

All the antisera (T, F1 and F2) had maximum titres against their respective syngeneic receptor antigen and this was defined as 100% cross-reactivity (Table 24, page 158). Both F1 and F2 showed little cross reactivity (1% and 0.35% respectively) against AChR from Torpedo electric organ. Similar relative activities were demonstrated by passive haemagglutination (Table 25, page 159). Antiserum T exhibited slight cross-reactivity (0.7%) in RIA against human AChR. The antisera F1 and F2 showed greater

cross-reactivity (12.5% and 17.0% respectively) with human AChR than with Torpedo AChR.

Subunit specificities of polyclonal antisera raised against Torpedo AChR were determined by immunoblotting (Methods, Section C2.2) when all four subunits ($\alpha, \beta, \gamma, \delta$) were found to be bound.

Table 24 Reactivities of rabbit antisera raised against Torpedo AChR and fetal calf AChR towards detergent-solubilised AChR from human and fetal calf skeletal muscle and Torpedo electric organ.

Antiserum Code	Immunogen	Titres against various AChR-[^{125}I]-α-BGT preparations x 10^{-9} M		
		Torpedo	Human	Fetal Calf
T	Torpedo AChR	1900(100%)	15(0.7%)	N.D.(-)
F1	Fetal Calf AChR	3.5 (1%)	40(12.5%)	320(100%)
F2	Fetal Calf AChR	2.5 (0.35%)	120(17.0%)	700(100%)

Figures in brackets represent the % cross-reactivity of each antiserum with the stated antigen, where 100% cross-reactivity is the titre of an antiserum using syngeneic antigen.
N.D. = Not determined

2. Antigenic characterisation of anti-(AChR) Mabs

The antigenic characterisation of the anti-(AChR) Mabs B11, C7, C11, E8 and E11 was studied by using a number of methods. The titres of Mab ascitic fluid and Mab supernatants were determined as was the cross-reactivity of the antibodies with AChR from different species. The subunit specificities of the Mabs and the affinities of the antibodies for AChR were also examined.

Table 25 Comparison of reactivity of polyclonal antisera towards Torpedo AChR by RIA and Passive haemmagglutination

Antiserum	RIA titre (nM)	Haemagglutination Titre
T	1900	1: 65536
F1	3.5	1: 256
F2	2.5	1: 8
Control serum	N.D.	1: 2

Rabbit anti-(Torpedo AChR) antiserum (T) and rabbit anti-(fetal calf AChR) antisera (F1) and (F2) were titrated against Torpedo AChR coated SRBC (Methods, Section B2.3). RIA titres were from Table 24, page 158. Control serum (NRS) had no detectable (N.D) titre in the RIA. Titration of antisera against BSA/coated SRBC showed agglutination in the first well only.

2.1 Titres and cross-reactivities of Mab ascites fluid and culture supernatants

2.1.1 Torpedo, fetal calf and human AChR antigens.

Hybridoma cells secreting the monoclonal anti-Torpedo (AChR) antibodies B11, C7, C11, E8 and E11 were used to produce ascitic fluid in congenic mice (Methods, Section B4.10). The titres of the ascitic fluids were determined in order to assess their cross-reactivities (see Table 26, page 160). The titres were expressed as nmoles of [^{125}I]-α-BGT binding sites bound per litre of ascites fluid.

The titres of the Mabs against Torpedo AChR ranged from 0.03-301nM. The cross-reactivites of the Mabs against AChR from mammelian sources varied greatly. Mab E11 had a low titre against Torpedo AChR but cross-reacted well with AChR from fetal calf and human muscle. All the Mabs reacted to a greater extent with fetal calf AChR than with human AChR.

Table 26 : Properties of mouse Mabs to Torpedo AChR

Mab	Immunoglobulin class	Subunit Specificity	Titre (nM) for AChR from Torpedo	Fetal Calf	Human Muscle
B11	IgG1	α, β	180	4.5	1.51
C7	IgG1	*	110	0.6	0.004
C11	IgG1	α, β	139	0.75	0.12
E8	IgG1	α	301	2.32	0.35
E11	IgG1	*	0.034	0.008	0.006

All titres are of ascites fluid using 0.5nM of [^{125}I]-α-BGT labelled antigen.

* Binding was not observed using Western blotting (Methods, Section C2.2).

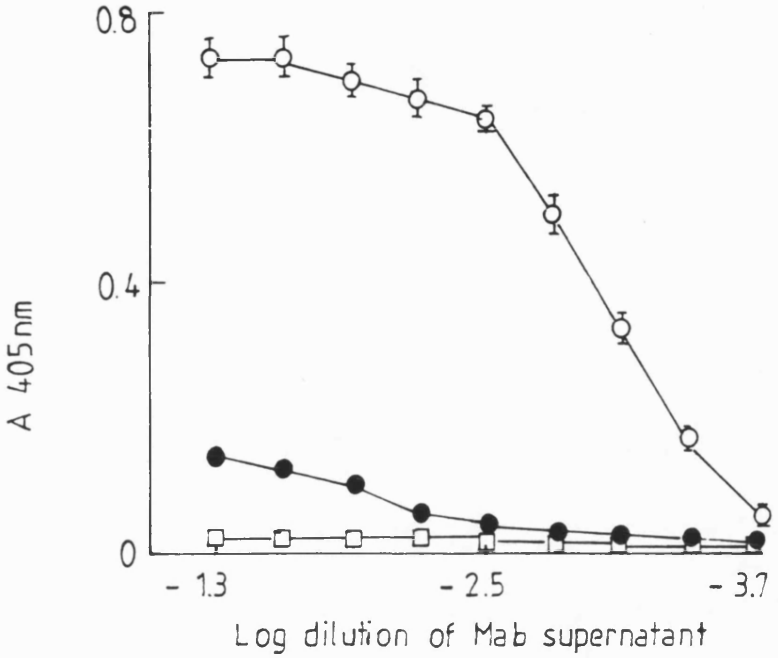
Three methods were used to determine the titres of culture supernatants. These were :- RIA (Lindstrom, 1977, Methods, Section B2.1), passive haemagglutination assay (Methods, Section B2.3), and ELISA (Methods, Section B2.2). The results are given in Table 27 page 163.

The ELISA proved to be a sensitive assay system and enabled titration curves of Mab binding against Torpedo AChR to be constructed. Figure 23 (page 162) gives examples of titration curves obtained for the Mab supernatant C7 and for a low titre supernatant Mab E11. The background absorbance determined by using normal culture medium or an irrelevant Mab supernatant was negligible. Mab C7 had a detectable titre of 1 in 2560 whereas Mab E11 had a titre of 1 in 160. The minimal detection level by the RIA for Mab C7 was 1/160 dilution, showing the ELISA system to be a more sensitive assay system. Mab supernatants were also titrated against Torpedo AChR coated SRBC. However, there proved to be little correlation between the agglutination and RIA titres.

2.1.2 Cultured rat myotube AChR as antigen

The cross-reactivities of Mab supernatants was carried out by using an ELISA technique with AChR expressed by rat myotubes after 7 days in culture (Methods, Section C 2.1.1c). At this stage, cultures were assayed for [^{125}I]-α-BGT binding sites by using an excess of radioligand (Methods, Section C2.1.1b). The mean specific binding of [^{125}I]-α-BGT was 52 fmols [^{125}I]-α-BGT binding sites/culture well (mean from two independent cultures) and is within the range quoted by Childs, (1985).

Figure 23 Titration of Mab supernatant by Torpedo AChR ELISA system



Mab supernatants (-O-, C7; -●-, E11) and normal culture medium (-□-) were titrated against AChR by ELISA. Each point is the mean of triplicate wells and the standard error shown for Mab C7 is representative for the rest of the assay.

Table 27 Titration of mouse anti-(AChR) Mab supernatants

Antibody	Radioimmunoassay Titre (nM)	Haemagglutination Titre	Titre by ELISA
B11	180	1:16	N.D.
C7	126	1:1280	1:2560
C11	139	1:16	N.D.
E8	301	1:64	N.D.
E11	45	1:8	1:160

Titre detected by RIA (Lindstrom, 1977) and expressed in terms of α-BGT binding sites.

Haemagglutination titre is given as the dilution at the end point.

ELISA titre is given as the dilution at which an absorbance level greater than that of the control value is obtained (see Figure 23, page 162).

N.D. Not determined using the same batch of Mab supernatant.

Cross reactivity was determined by measurement of absorbance after incubation with anti-(AChR) Mab or control Mab supernatant, followed by incubation with peroxidase-conjugated antibody and subsequent development using TMB as substrate (Methods, Section C2.1.1c). Three of the Mabs, B11, C11 and E8 showed varying degress of cross-reactivity with the nAChR expressed by rat myotubes, whereas the Mabs C7 and E11 gave OD readings similar to those of the control Mab (see Table 28, page 164).

Table Cross-reactivities of Mabs with Rat Myotube Cultures by ELISA

Test		Absorbance 450nm
Control Mab		0.05 ± 0.04
Normal culture medium		0.07 ± 0.03
Mab	B11	0.28 ± 0.08
	C7	0.13 ± 0.03
	C11	0.31 ± 0.02
	E8	0.20 ± 0.04
	E11	0.09 ± 0.02

Results are the mean ± SE of three separate culture wells prepared from the same culture. Absorbance from substrate alone has been subtracted.

2.2 The subunit specificity of anti-(Torpedo AChR) Mabs

The subunit specificities of the Mabs B11, C7, C11, E8 and E11 were determined by immunobinding of ascitic fluid following electro transfer of Torpedo AChR protein from SDS gels onto nitrocellulose (Methods, Section C2.2). The positions of the stained bands on the immunoblots were compared directly with those of a reference strip of nitrocellulose containing electroeluted AChR stained with india ink (Methods, Section C 2.2b).

It was evident that Mab E8 bound to the α-subunit, whereas the Mabs C11 and B11 recognised both the α and β subunits. These Mabs also bound to very high molecular weight material (Mr>

120Kd). No binding was observed with Mabs C7 and E11. The controls , NMS and NRS showed no binding. The results are summarised in Table 26 (Page 160).

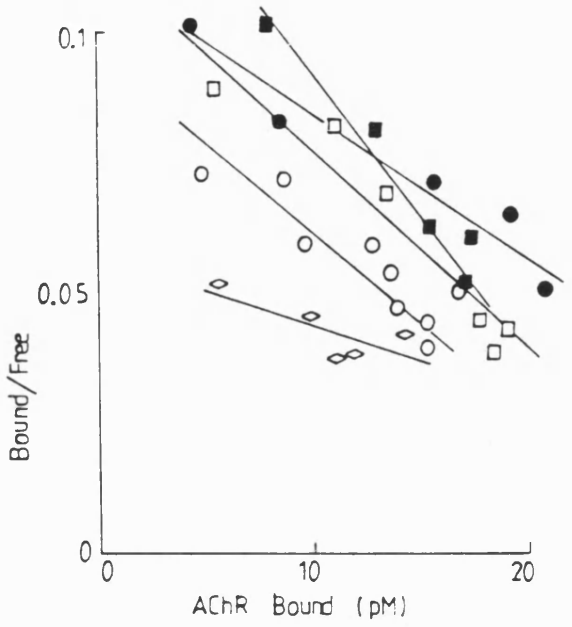
2.3 Immunoglobulin subclass of the Mabs

The immunoglobulin subclass of the Mabs (B11, C7, C11, E8, E11) was determined by an immuno-dot binding assay (Methods, Section B4.12). Standard anti-immunoglobulins (IgG1, G2a, G2b, G3 and M) were spotted onto nitrocellulose filters, followed by immunoblotting with the 5 Mabs plus controls NMS and a Mab AJ3 of known immunoglobulin subclass. All the Mabs were shown to be IgG1 subclass. As expected NMS reacted with all of the standards and AJ3 reacted with IgM.

2.4 Affinities of the Mabs towards AChR

The affinities of the Mabs were determined by incubating Mab supernatants with 0.08-0.4nM [^{125}I]-α-BGT labelled Torpedo AChR followed by immunoprecipitation with second antibody (Methods, Section C2.3). The binding curves were analysed by Scatchard analysis (Figure 24 , Page 166) to obtain values for kd. The values obtained are summarised in Table 29 (Page 166) and were in the range 1.76-7.5 x 10^{-10}M. These values are within the ranges quoted for anti-(Torpedo marmorata AChR) Mabs (0.1-7.6 x 10^{-10}M; Whiting et al., 1986) and anti-(Electrophorus electricus AChR) Mabs (3.2-30 x 10^{-10}M; Tzartos et al., 1981).

Figure 24 Scatchard plots for the determination of the affinity of binding of Mab to Torpedo AChR



100 μL of culture supernatant of each Mab was used in all cases except for Mab E11 where 200 μLs of supernatant was used to obtain a greater range of precipitable counts because this supernatant had a low titre.

Table 29 Summary of the results obtained from Scatchard analysis

Mab	Symbol	Affinity Kd x 10^{-10} M	Correlation Coefficient
B11	■	1.76	0.97
C7	●	3.62	0.96
C11	○	2.7	0.96
E8	□	2.59	0.96
E11	◇	7.50	0.81

2.5 Interaction of Mabs with the cholinergic binding site on AChR determined by using inhibition and dissociation assays

Sera from patients with MG, which contain antibodies against self-AChR have been demonstrated to block the binding of α-BGT to AChR (Almon and Appel, 1975). Similarly, anti-(AChR) Mabs have been produced which can interfere with the binding of α-BGT or other cholinergic ligands to the AChR (references in Introduction, Page 22). Mabs directed against the cholinergic site may be useful tools in comparing the binding sites of the AChR from different species and different tissues. In order to determine whether any of the Mabs produced in this study recognised the cholinergic binding site of Torpedo AChR a combination of different methods was investigated.

2.5.1 Inhibition of [^{125}I]-α-BGT binding to AChR-coated SRBC

The study of the effects of Mabs on α-BGT binding to AChR was initially determined by using immobilised AChR in the form of AChR-coated SRBC (Method, Section C2.4a).

Three Mabs (B11, C11 and E8) gave no significant inhibition; similar values within 10% of the control (anti-BSA Mab) were obtained. Two Mabs, C7 and E11 inhibited [^{125}I]-α-BGT binding by 42% and 33% respectively, when 300μl culture supernatant was used in a total assay volume of 400μl (Table 30 and Figure 25 , page 168).

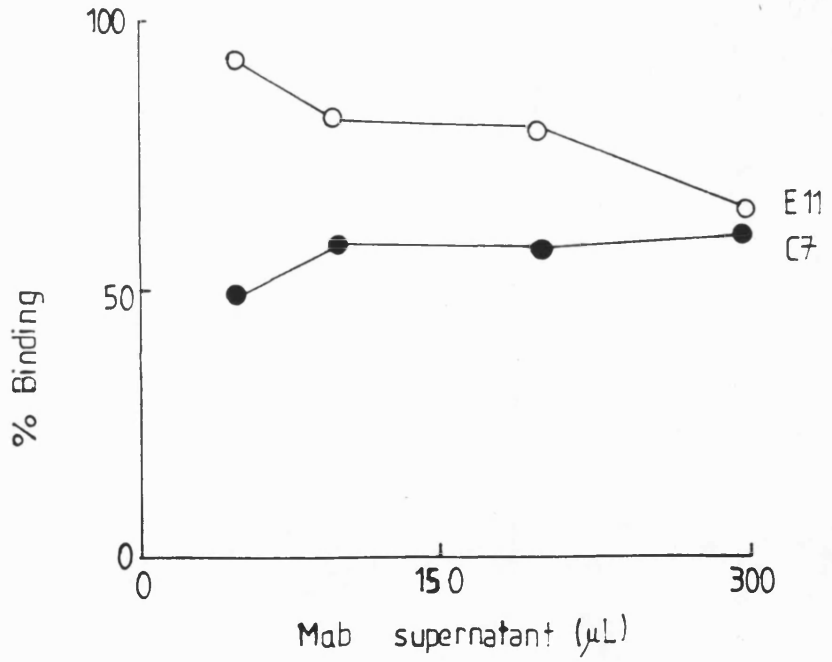
2.5.2 Inhibition of [^{125}I]-α-BGT binding to AChR using DEAE filter assay

To further investigate the inhibition of [^{125}I]-α-BGT binding,

Table 30 Inhibition of $[^{125}I]$-α-BGT binding to AChR coated SRBC by Mab

Mab supernatant	% inhibition of $[^{125}I]$-α-BGT binding
anti-BSA	0
B11	0
C7	42
C11	+ 10
E8	6
E11	33

Figure 25 Inhibition of $[^{125}I]$-α-BGT binding to AChR SRBC by increasing volumes of Mab supernatants C7 and E11



Increasing volumes of Mab supernatants C7 and E11 were used in the inhibition assay described in Methods, Section C 2.5.1.

to Torpedo AChR by Mab C7, the DEAE filter assay (Methods, Section C2.4b) was used. To ensure Mab excess, C7 supernatant was titrated with [^{125}I]-α-BGT-labelled AChR in an RIA procedure (Methods, Section B2.1) using Torpedo AChR (10fmols, equivalent to 28pM) prelabelled with a ten-fold excess of [^{125}I]-α-BGT. Saturable binding of labelled AChR by Mab C7 was obtained by using 20μl of supernatant (Figure 26, page 170). Hence, volumes of C7 antibody greater than 20μl should expose any inhibition of [^{125}I]-α-BGT binding to AChR. In order to increase the sensitivity of the assay, a 3-fold excess of [^{125}I]-α-BGT was used in place of the 10-fold excess usually used in toxin binding assays (Methods, Section A2.2).

Mab C7 inhibited toxin binding to AChR to a maximum of 50% (Figure 27 , page 170). Control Mab, B11, and anti-(BSA) Mab showed no inhibition of toxin binding, in agreement with the previous study using AChR coated SRBC.

2.5.3 Effect of Mabs on the dissociation of the [^{125}I]-α-BGT - labelled AChR complex

The possibility that Mab C7 could also enhance the dissociation of [^{125}I]-α-BGT from AChR was examined by incubation of labelled AChR complex with a large excess of Mab C7 ascites fluid ($\sim$ 4 nmols) followed by removal and filtration of samples on DEAE filters at different time intervals (Methods, Section C2.4b). The results were expressed as the percentage of labelled AChR bound at zero time; non-specific binding was determined in the presence of BZQ. Ascites fluid from Mabs B11, C11 and E8 and NMS were used as controls. Mab E11 was not used

Figure 26 Titration of Mab C7 by RIA to ensure antibody excess for [^{125}I]-α-BGT DEAE filter inhibition assay

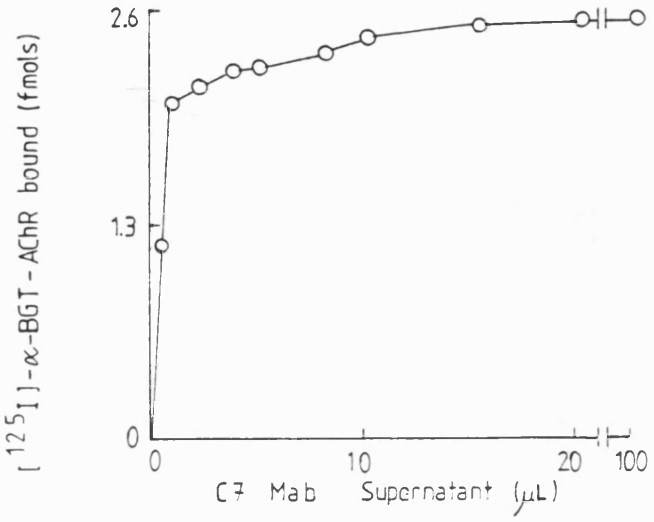
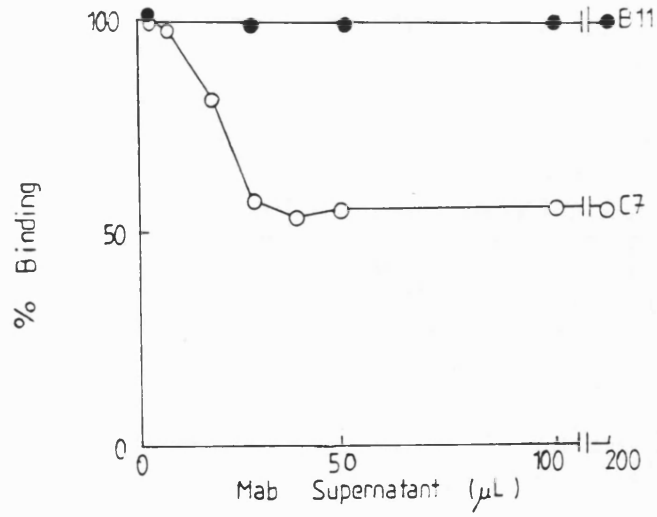


Figure 27 Inhibition of [^{125}I]-α-BGT binding by Mab by using the DEAE filter assay



because of the low titre of the ascitic fluid (Table 26 , page 160).

None of the Mabs examined caused dissociation of [^{125}I]-α-BGT-labelled AChR complex over the time period 0-18h.

2.6 Investigation of the interaction of Mab with the cholinergic binding site of AChR by ELISA

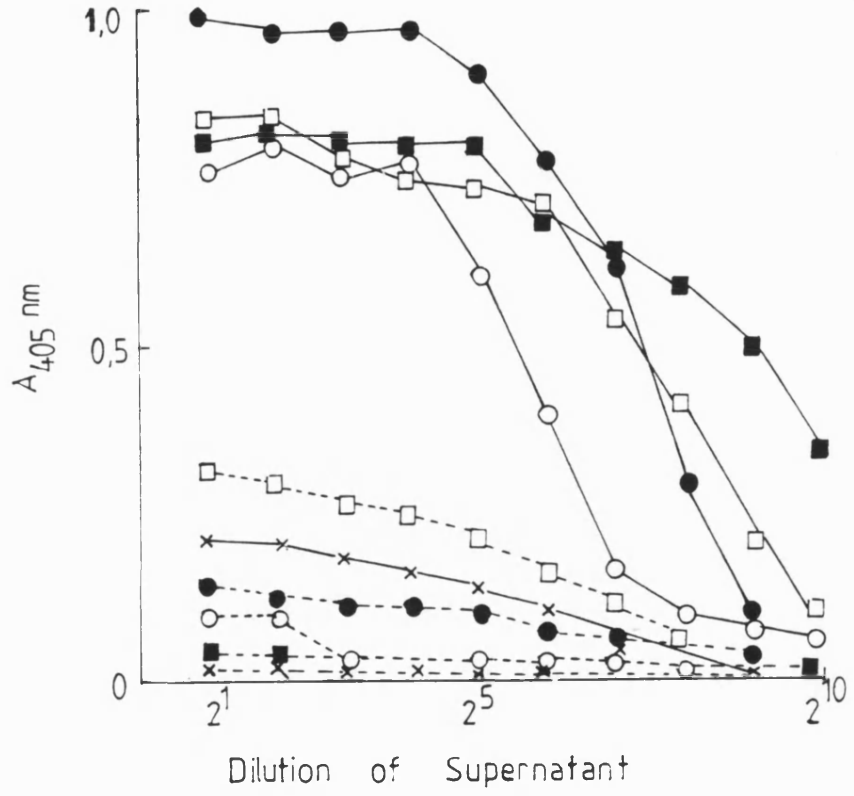
Adaptation of the basic ELISA method allowed a limited analysis of the interaction of Mab with the cholinergic binding site on AChR. The first study uses immobilised α-BGT to orientate AChR in such a manner that only sites remote from the cholinergic binding site are exposed. The second study examined the effect of various cholinergic ligands on the binding of Mab to immobilised AChR (Methods, Section C2.5).

2.6.1 The effect of precoating plates with α-BGT on the titration of Mabs against immobilised AChR

Serial dilutions of Mab supernatant were incubated with ELISA wells which had been coated directly with Torpedo AChR or with α-BGT followed by AChR and the results compared (see Figure 28 page 172). Mabs E8, C11, C7 and E11 showed negligible binding to Torpedo AChR when this was attached by α-BGT to ELISA plates, whereas the binding of Mab B11 was only partially reduced.

To determine whether the reduced binding of Mab to α-BGT-AChR coated wells was due to less AChR actually being bound by this method, the amount of 'non-bound' AChR was pooled from twenty wells coated with AChR either directly or indirectly by α-BGT. The [^{125}I]-α-BGT binding activity of the two pools was

Figure 28 Comparison of titration curves of Mab supernatants on AChR coated and α-BGT-AChR coated ELISA wells



ELISA wells were coated with Torpedo AChR directly (——) or by pre-coating plates with α-BGT (- - -) and incubated with serial dilutions of Mab supernatant (□ B11; ■ C7; ○ C11; ● E8; × E11). The results are a composite of individual experiments with each Mab supernatant.

Cross-reactivity of supernatants with wells coated with α-BGT only was negligible. Similarly no cross-reactivity was observed with irrelevant Mab supernatant.

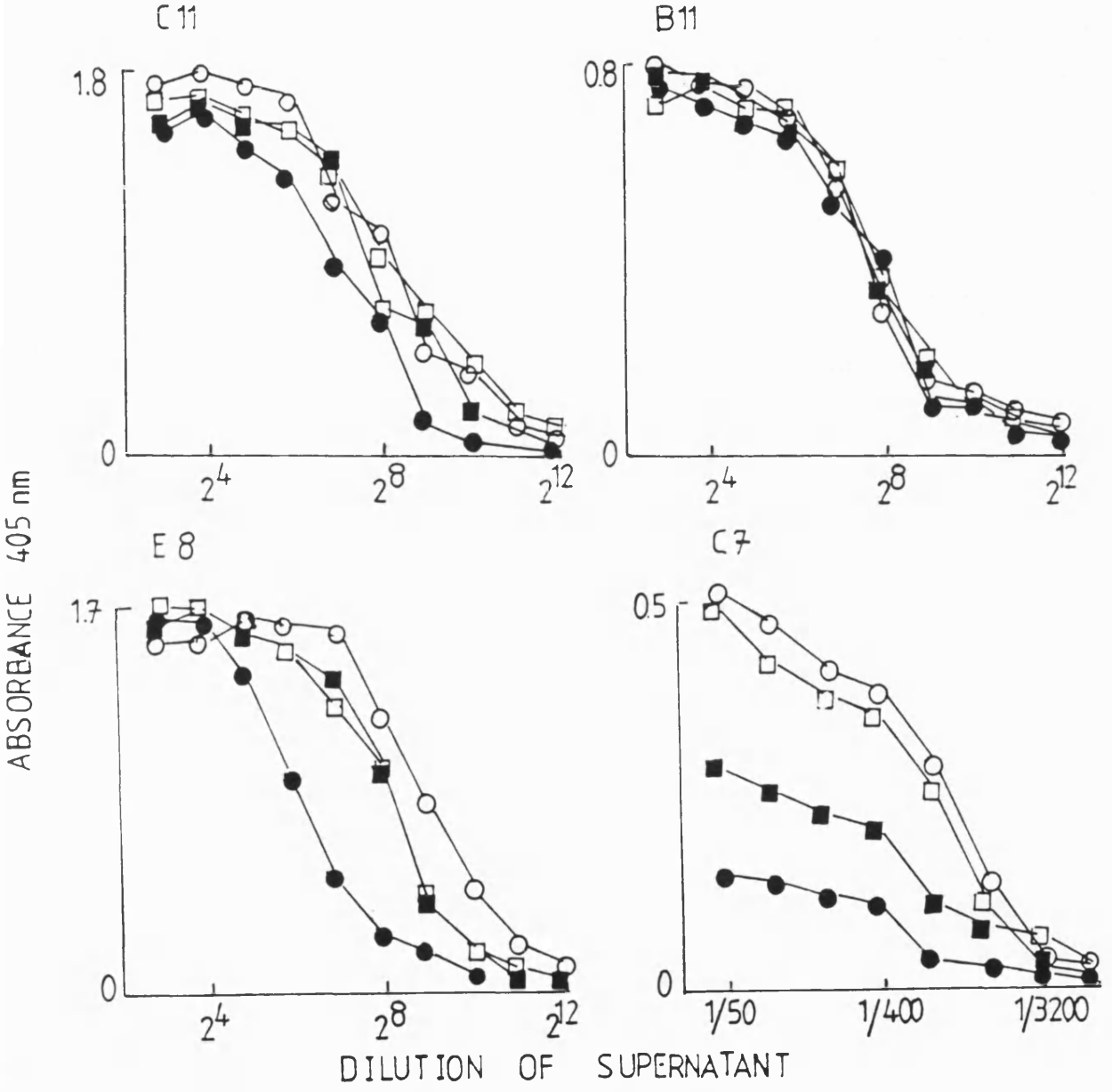
determined by DEAE filtration assay (Methods, Section A2.2). There was only a negligible difference (~3%) between the amount of 'non-bound' AChR present in the two pools, indicating that the two coating procedures bind approximately similar amounts of AChR.

2.6.2 Effect of cholinergic ligands on binding of Mab to immobilised AChR

The effects of the cholinergic ligands, carbachol, d-tubocurarine and BZQ on the binding of Mab supernatants to immobilised AChR were examined by using an ELISA (Methods, Section B2.2). The resulting profiles are given in Figure 29 (page 174) Mab E11 was not examined because of its low titre (Results, page 162).

The binding of Mabs B11 and C11 were unaffected by the presence of cholinergic ligands, whereas the binding of Mab C7 to immobilised AChR was decreased. The most effective inhibitor was BZQ followed by d-tubocurarine then carbachol. The binding of Mab E8 was decreased only by the presence of BZQ (Figure 29 , page 174).

Figure 29 Effect of cholinergic ligands on binding of Mab to immobilised AChR



Torpedo AChR immobilised on ELISA plates was incubated with serial dilutions of Mabs (C11, B11, E8 and C7) in the presence of the cholinergic ligands, carbachol (-□ -), d-tubocurarine (-■ -) and benzoquinonium (-● -). Control values (-○ -) were obtained in the absence of ligand. Each point is the mean of six wells and each set of curves is representative of two experiments.

DISCUSSION

Polyclonal antisera were raised against AChR from Torpedo marmorata and from fetal calf muscle. A small library of monospecific antibodies, raised against Torpedo marmorata was also established. Both polyclonal antisera and Mabs were characterised with respect to titre and cross-reactivity. In addition the Mabs were further studied with respect to affinity, subunit specificity and interaction with the cholinergic binding site of the AChR.

The usefulness of polyclonal and monoclonal anti-(AChR) antibodies as probes of AChR-related molecules is, of course, dependent on the properties of the antibodies themselves. A major factor concerns their cross-reactivity; ie., do the antibodies recognise determinants which are common to AChRs from many different species or only those carried by the AChR type to which they were raised. The former property, rather than the latter, suggests binding to conserved regions of the receptor protein and hence such antibodies provide much more useful probes for detecting AChR from other tissues.

AChR prepared from fetal calf muscle and Torpedo electric organ was able to stimulate the production of a population of antibodies which recognised determinants found on AChR from xenogeneic sources. This finding is consistent with the highly conserved nature of the AChR from various species, as revealed by significant homologies in the primary structures

(Kubo et al., 1985, see Figure 30 page 191), similar (but not identical) molecular masses of the subunits (Einarson et al., 1982) and similar pharmacology and electrophysiological properties (Anholt et al., 1984). The greater cross-reactivity shown by anti-(fetal calf AChR) antisera F1 and F2 with AChR from human muscle than with Torpedo AChR may represent the closer phylogenetic relationship of the bovine and human receptor types. Cross-reactivities of anti-(Torpedo AChR) and anti-(fetal calf AChR) antisera similar to those found here have also been shown by other workers (Lindstrom et al., 1978a, 1979).

An RIA procedure, using [^{125}I]-α-BGT labelled AChR, was used to assay the antisera. One disadvantage of this method is that it does not detect antibodies directed against the α-BGT binding site, although such antibodies appear to be rare in polyclonal anti-AChR sera (Karlin et al., 1978; Whiting et al., 1983, Lukas, 1984) suggesting that the α-BGT binding site is not highly immunogenic. One interesting exception, appears to be the high proportion of anti-'site' antibodies in polyclonal antisera raised to chick muscle AChR (Mehraban et al., 1984) although the significance of this is not clear.

Haemagglutination of AChR-coated SRBC provided a simple alternative assay that is able to detect anti α-BGT binding site antibodies. This method has been successfully used to detect and characterise anti-(AChR) Mabs in a study by Gomez et al.,(1979). Haemagglutination by antibody is dependent on the cross-linking

ability of the test antibody. Polyclonal antisera contain, by definition, antibodies directed against multiple antigenic determinants and so are well suited to this assay. Correlation between the titres obtained by RIA and haemagglutination assays was not surprising as both methods rely on the recognition of a large number of AChR epitopes by the polyclonal antisera. Additionally the correlation found suggests that few antibodies within the antibody population were directed against the cholinergic binding site of AChR.

Mabs are directed to single antigenic determinants and cross-reactivity is dependent on the presence of a common antigenic determinant, ie: one would expect to see either 100% reactivity or none at all. All the Mabs produced against Torpedo AChR cross-reacted with AChR from fetal calf and human muscle indicating that the Mabs recognise AChR epitopes common within species and this finding again reflects the extensive homology between the AChR receptors as shown by sequencing studies (see Kubo et al., 1985). Another important factor in determining the extent of cross-reactivity observed is the binding affinity between the Mab and the cross-reacting epitope. All the Mabs showed single site high affinity binding to Torpedo AChR (see later for discussion), hence the observed low titres of the Mabs against AChR from mammalian sources (Table 24, page 158) must reflect lower affinity binding and it would be of interest to titrate out all the low affinity antibody binding sites using high concentrations of labelled antigen to verify this. A comprehensive study by Tzartos et al., (1981) found

that Mabs raised against denatured AChR from Electrophorus electricus recognised receptors from other species but with lower binding affinities. The decreased binding affinities may be due to subtle differences in conformation resulting from point amino-acid differences between AChRs from various sources.

Although the AChR populations from fetal calf and human muscle are likely to contain a mixture of both JR and EJR (see Section A for discussion) this is unlikely to affect the titres observed since the concentration of antigen was in excess. However preferential reactivity of the Mabs to one of these receptor forms may have been present. Souroujon and co-workers (1985) described four anti-(Torpedo AChR) Mabs which showed preferential binding to the denervated form (EJR) of receptor of rat muscle. This again may be the consequence of different binding affinities due to slight differences in structure of the two receptor types and parallels the preferential binding of myasthenic sera with EJR (Weinberg and Hall, 1979, Dwyer et al., 1981).

Antigenic differences between normal and denervated AChR have also been found using Mabs to AChR from human muscle (Whiting et al., 1986) using, in part, evidence from staining of frozen sections of human endplates and this indeed may represent a useful future approach for characterisation of the Mabs described here. In this study, AChR expressed by cultured rat myotubes was used as a source of EJR and indeed three of five Mabs showed cross-reactivity.

These recognition properties could be further exploited in future studies which might investigate whether the antibodies cause increased internalisation of AChR or are capable of complement mediated cell lysis (see Tzartos and Powitz, 1986 and Childs et al., 1985 for the description of techniques). All of the antibodies produced were IgG1; this subclass also formed a major proportion (75%) of the Mabs produced by Whiting et al., (1985) against AChR from Torpedo marmorata and represents 38% of the Mabs produced in rat against eel, Torpedo and calf AChR (Tzartos et al., 1985). Such antibodies should be able to fix complement and hence they may be of use in lysis studies.

In contrast to the correlation observed between RIA and haemagglutination titres using polyclonal antisera, there was no apparent correlation using Mabs. Haemagglutination titres are dependent on the cross-linking abilities of the antibodies concerned (as discussed previously), and those with high titres (Mabs C7 and E8) may show greater degree of intermolecular, as apposed to intramolecular cross-linking. The nature of cross-linking is in turn dependent on the subunit specificty of the Mab involved. Mab E8 showed specificity for the α-subunit, whereas the subunit specificty of Mab C7 is not confirmed, but is most probably α-subunit specific (for reasons more fully discussed below). These antibodies may bind AChR molecules intermolecularly whereas the other Mabs, also having α-subunit specificities (see Table 26) but with low haemagglutination titres, may bind AChR intramolecularly. These proposals could be verified by the analysis of Mab-AChR

complexes by sucrose density gradient centrifugation as described by Conti-Tronconi et al., (1981a).

The Mabs C7 and E11 were unable to recognise AChR subunits that had been exposed to SDS and transferred to nitrocellulose in the Western blot procedure. It is possible that these Mabs bind to highly conformationally dependent epitopes, as the subunits should be largely renatured following electrotransfer, rather than sequential antigenic determinants. Similar conformationally dependent Mabs have been described by Tzartos et al., (1981). These determinants may be formed by amino acids from non-adjacent parts of a subunit or parts of other subunits. These same antibodies were also capable of partially inhibiting [^{125}I]-α-BGT binding to AChR and it is probable that their specificity lies in part, if not all, on the α-subunit of the AChR.

It may be that the Mabs bind with very low affinity to denatured AChR (Tzartos and Lindstrom, 1980) and incubation with denatured AChR subunits on nitrocellulose paper in this study was carried out overnight in the hope of detecting low affinity binding, however without success. The use of the more sensitive method of radiography, rather than the enzyme detection system, may be of benefit here (Lindstrom, 1984). Analysis of the subunit specificity of these Mabs by a method which does not involve subunit SDS denaturation may provide more definitive data. An ELISA system in which purified subunits are bound to microtitre plates has been described (Lindstrom, 1984), but the sensitivity

of this method is affected both by how the subunits bind to the plastic and by the purity of the subunit preparation. Inspite of these drawbacks, this method has been used with success for screening Mabs (Tzartos et al., 1986). Another alternative is to immunoblot Cleveland type peptide maps (Lindstrom, 1984).

Mabs C11 and B11 reacted with determinants on two different subunits, α and β. This particular dual specificity appears to be uncommon in the literature. However, Souroujon et al., (1983) and Tzartos and Lindstrom (1980) have each reported similar Mabs. Interestingly, in the latter case, the Mab was obtained after the immunisation with native AChR and a purified subunit preparation. In early studies, anti-(Torpedo α-subunit) antiserum was shown to recognise Torpedo β-subunits to a greater extent than the γ and δ subunits (Lindstrom et al., 1979); a finding that is consistent with homology data (Kubo et al., 1985; see also Figure 30). Hence, the Mabs C11 and B11 may bind to homologous regions of the α and β subunits. Mabs have also been prepared which recognise homologous determinants on γ and δ (Froehner et al., 1983) and even on all four subunits (Gullick & Lindstrom, 1983).

Three of the fives Mabs produced were shown to recognise the α-subunit (Mabs B11, C11 and E8). This high proportion of Mabs recognising the α-subunit is not unexpected, as the most immunogenic region of the AChR, the MIR, is located on the α-subunit (see Introduction, Section 3). Indeed, other

workers have found that about half of the antibodies produced by immunisation with native receptor, are directed at this site (Tzartos and Lindstrom, 1980; Tzartos et al., 1981). Immunisation with denatured receptor results in a totally different antibody reactivity pattern and the resultant antibodies show preferential binding to the cytoplasmic surfaces of the subunits (Froehner et al., 1983; Sargent et al., 1984). Because the Mabs B11, C11 and C8 reacted with AChR expressed by rat myotubes, it is evident that they recognise extracellular determinants. This was confirmed by the ELISA in which all the Mabs apparently bound to AChR domains which were in the same orientation as the α-BGT binding site. Mab B11, however, appeared to bind also to determinants on the periphery of the AChR molecule as its binding was only partially blocked by coating the plate first with α-BGT.

It is not possible, from the data, to say whether the Mabs B11, C11 and E8 are directed at the MIR without carrying out competitive binding experiments (see Lindstrom , 1984 for experimental details). The MIR is highly conserved and antibodies directed at this determinant are capable of passively transferring EAMG. None of the Mabs produced in this study appeared to induce myasthenic symptoms in mice during the production of ascitic fluid. However, it was difficult to distinguish the symptoms of EAMG from those of the trauma caused by tumour formation. The MIR is highly conserved and the well-defined anti-MIR Mab 35 (Tzartos et al., 1981) has been used to detect nAChR at synapses on chicken ganglionic neurones

(Swanson et al., 1983). This study highlighted the potential use of such Mabs as probes since the conventional receptor probe α-BGT failed to detect the receptors at these regions (see Introduction, Section 3.6).

The estimation of the affinity of Mabs towards AChR is also complicated by their subunit specificities. Mabs that are capable of intermolecular cross-linking will give lower Kd values, suggesting higher affinity, than a Mab which binds intramolecularly. Torpedo AChR is known to exist as dimers, which further complicates the determination of Kd. Nevertheless the binding studies carried out here provided a relative estimate of the binding affinities of the Mabs described.

The Mabs were originally screened by using an ELISA procedure and it was possible that antibodies could have been selected with specificities directed at the α-BGT binding site of AChR. However, all the Mabs had detectable titres by the RIA procedure, using [^{125}I]-α-BGT labelled AChR. Despite this, two of the five Mabs (C7 and E11) inhibited, to a maximum of 50%, [^{125}I]-α-BGT binding to AChR. There are reports of such antibodies in MG sera and in polyclonal anti-AChR sera (see Harrison and Behan, 1986 for review) although their proportion is relatively small (Whiting et al., 1983). There are however several examples of a similar high incidence of site-directed Mabs (Gomez et al., 1979; James et al., 1980; Watters and Maelicke, 1983; Mehraban et al., 1984; Whiting et al., 1985) although not reported by Tzartos and Lindstrom, 1980. Such Mabs

can be useful in studying the ligand binding site of the AChR (examples Donnelly et al., 1984; Mihovilovic & Richman, 1984; Fels et al., 1986) or indeed may affect other functions of the AChR, such as channel gating (Blatt et al., 1986). Modifications of the RIA procedure (Whiting et al., 1985) have been described, as have assays capable of selecting function-affecting antibodies (Watters and Maelicke, 1983; Fels et al., 1986).

The Mabs C7 and E11 do not necessarily bind at the α-BGT binding site; it may be that the antigenic determinant is situated close enough to the α-BGT binding site, such that binding of the Mab sterically hinders the binding of α-BGT. The arm of a bound Mab has a reported diameter of approximately 35$\mathring{A}$ and can cover an area of 10nm^2 (Tzartos et al., 1981). It is also possible that the binding of the Mab results in a conformational change in the AChR which leads to occlusion of the α-BGT binding site. Such Mab-induced changes in AChR conformation have been proposed by Maelicke et al., (1986), who suggest that Mabs cannot be considered as static surface markers but rather as ligands which can induce conformational changes through allosteric networks.

Partial inhibition by Mabs of α-BGT binding has also been seen by other workers (Whiting et al., 1985; James et al., 1983; Mehraban et al., 1984). These findings could indicate the presence of two different sites on the same AChR molecule or two different populations of AChR. There is no evidence for this latter point. The α-subunits of AChR from Torpedo have the same primary sequence (Noda et al., 1982;

Sumikawa et al., 1982b; Devillers-Thiery et al., 1983) but because the AChR is an asymmetric molecule the two α-subunits have slightly different environments (Kistler et al., 1982; Kubalek et al., 1987; see also Introduction, Figure 3). This may explain the different antigenic properties of the two α-subunits, as suggested by the production of antibodies directed at one rather than both α-BGT binding sites. Of interest, here, is that the ligand binding sites of the AChR have also been shown to be different; having a low affinity non-glycosylated site and a high affinity glycosylated site (Anholt et al., 1984).

The use of SRBC as a solid support for examining the inhibition, by Mab, of α-BGT binding to AChR was found to be a useful and rapid technique. It is, however, improbable that total inhibition of [^{125}I]-α-BGT binding, by any Mab, would have been observed by using this method. The coating procedure could lead to several different situations, (i) the target antigenic determinant could be modified by binding to the SRBC; (ii) the coating procedure could destroy or hide the antigenic determinant or (iii) the α-BGT binding site or (iv) both. Total inhibition would only be observed if both α-BGT binding sites were readily accessible to the Mab and α-toxin. It would be interesting to carry out the haemagglutination assay in the presence of excess α-BGT. A similar approach has been used by Gomez et al., (1979) to study the effect of cholinergic ligands on the binding of Mabs to AChR coated SRBC. However, in these studies it should be remembered that the α-BGT is a large

molecule (MWt 8,000) and could occlude many antigenic epitopes on the receptor other than or including the α-BGT site, which is known to be located between the amino acid residues 125-196 on the α-subunit (Wilson et al., 1985; Mulac-Jericevic and Atassi, 1986; Neumann et al., 1986). None of the above techniques can definitively prove Mab binding at the α-BGT binding site and other techniques such as Cleveland peptide mapping or competitive binding methods such as those used in the study by Whiting et al., (1985) may more precisely pin-point the regions recognised by these Mabs.

The α-BGT binding region represents a portion of the cholinergic ligand binding region of the AChR and several groups have reported antibodies that cross-react at the transmitter binding site rather than at the α-BGT binding site (Almon et al., 1974; Vincent, 1980; Drachman et al., 1981; Fulpuis et al., 1980; Mochly-Rosen & Fuchs, 1981). It would, accordingly, be interesting to use the Mabs C7 and E11 in functional assays such as ion flux studies to study their effects (see Souroujon et al., 1983). Interestingly, a Mab, with non-cholinergic site directed properties, has been reported that interferes with agonist-induced ion flux (Donnelly et al., 1984).

Clearly, as wide a range of screening procedures as possible should be used in order to detect antibodies with such novel properties.

Although Mabs C7 and E11 showed limited but detectable cross-reactivity with AChR from fetal calf and human muscle,

they did not recognise AChR expressed by rat myotubes. This finding deserves more attention, perhaps using fluorescent staining techniques. It does suggest, however, that the region of the α-BGT binding site has both conserved and non-conserved regions, as found by other groups (James et al., 1983; Watters and Maelicke, 1983; Mehraban et al., 1984; Whiting et al., 1985).

The DEAE filter assay provided an alternative and well characterised assay system for the further study of Mab C7. Using this assay, and by ensuring Mab excess over the antigen concentration, it would have been posible to determine whether the Mab was capable of blocking all, (unlikely in this study since the Mabs could precipitate AChR saturated with [^{125}I]-α-BGT or only a fraction of [^{125}I]-α-BGT binding sites. The results of the assay actually carried out confirmed the findings from the AChR coated SRBC assay for Mab C7 . This assay system also provided a means of studying the possibility of dissociation, induced by Mab, of the [^{125}I]-α-BGT labelled AChR complex. Such antibodies are extremely rare and must possess one or both of the following properties :- a higher affinity for the α - BGT binding site than α-BGT itself (unlikely since α-BGT binds in an almost irreversible fashion) or have the ability to interact with a site near to the α-BGT binding site in such a way as to induce a conformational change in the AChR such that the affinity of the α-BGT site for α-BGT is reduced. None of the antibodies studied here possessed this property. Mehraban et al., (1984) has described a Mab which is

capable of inducing the dissociation of [^{125}I]-α-BGT labelled AChR complex and IgG or serum from certain myasthenic patients have also shared this characteristic (Barkas & Simpson, 1982).

The Mab E11 proved to be somewhat anomalous in that it had low but detectable titres as assayed by RIA, ELISA and haemagglutination methods. This antibody also bound with high affinity to the [^{125}I]-α-BGT labelled AChR complex. One would expect that a Mab directed at a region near, or at the α-BGT binding site would show much lower precipitation of AChR saturated with [^{125}I]-α-BGT. This would appear to hold true in the RIA for this Mab. However, Mab C7 does not share these properties showing relatively high titres in all the assays used. The finding that Mabs behave differently under different assay conditions is not unusual (Lukas 1984) and, indeed, some Mabs have shown reduced affinity for AChR immobilised on poly-(vinyl chloride) microtitre wells (Watters and Maelicke, 1983; Lukas, 1984) relative to their affinity for detergent solubilised antigen in the immunoprecipitation assay. Overall, the present findings indicate that the Mabs C7 and E11 do not bind to the same antigenic determinant on the AChR. It may be worthwhile pursuing the study of the Mab E11 using ammonium sulphate cuts of culture supernatant, ascitic fluid or purified antibody to study the effects of higher concentrations on the inhibition of α-BGT binding to AChR by this Mab.

Competition, by cholinergic ligands, of Mab binding to AChR was studied by using the ELISA method. This provided an alternative

procedure to the commonly used precipitation assay with labelled toxin-receptor complexes (James et al., 1980; Whiting et al., 1985) and had been previously used by Watters and Maelicke (1983). The use of this kind of approach is, however, complicated by the affinities of the antibodies studied. Here, all the Mabs used had higher affinities (range $\sim$ nM) for the AChR than the cholinergic ligands used to compete (Table 31, page 191) leading to a situation where the higher antibody affinity binding could obscure the interaction of the cholinergic ligand with the AChR, hence reducing the chances of detecting anti-cholinergic ligand site directed Mabs.

Nevertheless, d-tubocurarine and BZQ partially competed with Mab C7 for binding to immobilised AChR. This finding and the ability of the Mab to inhibit α-BGT binding confirms the finding that this Mab recognises a site near to but not directly at the cholinergic binding site. Partial inhibition of binding of Mabs by cholinergic ligands has been reported by several groups (Gomez et al., 1979; James et al 1980 and Watters and Maelicke, 1983). Mabs have also been reported that are only inhibited by α-BGT (Gomez et al., 1979, James et al., 1980). The cholinergic ligands used here are smaller than α-BGT and hence inhibition by steric hindrance is not a major factor. Further investigations, using a wider range of cholinergic agents and making use of RIA methods may provide more information on the Mab C7.

The binding of Mab E8 to AChR was partially affected only by the nicotinic antagonist BZQ. One possible explanation of this is that the antibody binds to a determinant affected by conformational changes induced in AChR by the binding of the antagonist. Maelicke et al., (1986) have proposed overlapping sub-sites for cholinergic ligands on the basis of studies using Mabs. Indeed a Mab has been produced that can differentiate between d-tubocurarine and carbamylcholine binding sites (Mihovilovic and Richman, 1984). This provides further evidence that Mabs may provide insights into the mechanisms of receptors that are not accessible with other ligands.

Table 31

Dissociation constants of cholinergic ligands for AChR

Ligand	Kd
Carbamylcholine	4.5×10^{-5} M
d-tubocurarine	8×10^{-7} M
BZQ	data unavailable from the literature
α- bungarotoxin	2×10^{-9} M

Values taken from Changeux, 1981.

Figure 30 Homology matrix for the human, calf and *T. californica* acetylcholine receptor subunits

(Taken from Kubo et al., 1985)

	Human α	Calf α	Torpedo α	Calf β	Torpedo β	Human γ	Calf γ	Torpedo γ	Calf δ	Torpedo δ
α Human		97	79	37	40	33	32	35	34	34
α Calf	97		79	37	39	33	32	35	35	34
α Torpedo	80	81		35	40	32	31	34	36	34
β Calf	38	38	37		58	41	39	41	40	39
β Torpedo	41	40	42	59		40	40	41	40	41
γ Human	33	33	33	41	41		92	55	46	43
γ Calf	32	33	32	40	41	92		55	47	45
γ Torpedo	35	35	35	41	41	55	56		48	49
δ Calf	35	36	37	41	42	46	48	48		58
δ Torpedo	34	34	35	40	41	44	46	50	60	

SECTION D

PREPARATION AND CHARACTERISATION OF LYMPHOCYTES WITH RESPECT TO CHOLINERGIC BINDING SITES

METHODS

1. Isolation and characterisation of leucocyte populations and preparation of membrane fractions

1.1 Separation of PBL and granulocytes.

1.2 Lysis of erythrocytes.

1.3 Preparation of mouse thymocyte cell suspensions.

1.4 Giemsa staining for the examination of leucocyte populations.

1.5 Preparation of membrane fractions and detergent extracts of human PBL.

1.6 Preparation of P2 membrane fraction from rat brain.

2. Radioligand binding assays

2.1 [^{125}I]-α-BGT bindings assays.

2.1.1 Filtration assay
2.1.2 Centrifugation assay

2.2 [^{3}H]-QNB binding assays

2.3 [^{3}H](-)-nicotine binding assays.

2.4 Binding kinetics.

2.5 Inhibition studies using test drugs and anti-(AChR) monoclonal and polyclonal antibodies.

1. Isolation and characterisation of leucocyte populations and preparation of membrane fractions for radioligand binding assays

Human PBL and granulocytes were prepared for use in radioligand binding assays.

1.1 Separation of human PBL and granulocytes

PBL were separated from freshly-drawn heparinsed (10 IU/ml) venous blood (20-40ml), leucocyte enriched buffy coat and leucopheresis samples by density gradient centrifugation using a method described by Boyum (1968). Buffy coat samples (50ml) were obtained from healthy donors and supplied by the Blood Transfusion Service. These samples were routinely 18-20h old before being processed. Two leucopheresis samples from two MG patients were also processed. Buffy coat and leucopheresis samples were subjected to Dextran sedimentation prior to the density gradient centrifugation step in order to reduce the quantity of contaminating erythrocytes.

The buffy coat/leucopheresis sample was mixed with PBS containing 2% (w/v) Dextran (molecular weight 20,000-75,000, 16ml), and left to stand (1-2h, 37°C), until most of the erythrocytes had sedimented to the bottom leaving a white, turbid supernatant. This leucocyte rich fraction was removed, washed with PBS (200g, 10 min) and then resuspended in PBS (25ml). The suspension (5ml) was layered carefully onto Ficoll-Hypaque density gradients (4ml) and centrifuged (400g, 30min, 23°C). Heparinised venous blood samples were routinely diluted with an equal volume of PBS containing heparin (10

IU/ml) before being layered onto the separation medium. The mononuclear cells were recovered carefully from the Ficoll/plasma interface using a siliconised pasteur pipette, washed twice by centrifugation (200g, 10 min, 23°C), in PBS and finally resuspended in PBS (10-20ml).

Granulocytes were prepared by carefully harvesting the pelleted cells from the Ficoll-Hypaque centrifugation step. The cells were washed with PBS, centrifuged (200g, 5 min) and the contaminating red cells were removed by lysis with ammonium chloride (Methods, Section D1.2).

The viability of isolated PBL and granulocytes was estimated by Trypan dye exclusion (Methods, Section B4.1) and was routinely >90%. Erythrocyte contamination of the fractions was assessed by the difference in cell count between samples treated with Methylene blue (0.01% (w/v) in 1% (v/v) acetic acid) and Trypan Blue. When the erythrocyte contamination was greater than 2% the red cells were removed by ammonium chloride lysis.

1.2 Lysis of Erythrocytes

Two different buffers were used for lysing human and non-human erythrocytes using essentially the same procedure.

Contaminating human erythrocytes (0.1ml packed cell volume) were mixed with lysis buffer (0.9ml, 10mM potassium bicarbonate containing 0.155M ammonium chloride and 0.1mM EDTA) and incubated at room temperature for 5 min. The cells were then washed twice by resuspension in culture medium and

centrifugation (300g, 5 min). This procedure was repeated if the red cell contamination was still greater than 2%. For lysis of mouse erythrocytes, the cells were treated in the same way but the lysis buffer comprised 17mM Tris-HCl pH7.2 containing 0.144M ammonium chloride.

1.3 Preparation of mouse thymocyte cell suspensions

The thymus glands of 6 week-old CFLP mice were removed taking care to avoid any surrounding tissue. A suspension of thymocytes was prepared by gentle squeezing of the thymus with broad flat edged forceps into BSS comprising of 0.14M NaCl, 5.4mM KCL, 0.8mM MgSO4. 7H2O., 1.0mM CaCl2. 2H2O., 0.4mM KH2PO4 and 1.4mM Na2HPO4, pH7.2. A single cell suspension was obtained by passage of the resulting cells through a fine wire mesh. Contaminating erythrocytes were removed by lysis as described in the Methods, Section D1.2 and the cell viability, as assessed by Trypan dye exclusion, was >98%. The yield of thymocytes from each thymus gland was $1.80 + 0.47 \times 10^8$ (mean $\pm$ S.E., n=30).

1.4 Giemsa staining for the examination of leucocyte population

A nuclear stain, Giemsa, was used to study the morphological characteristics of fixed smears of human PBL.

Human PBL were prepared by density gradient centrifugation (Methods, Section D1.1) and were suspended in neat NRS and smeared onto clean microscope slides. NRS was used to protect the cells from damage during smearing. The slides were allowed to air dry, fixed in methanol (90% v/v) for 2 min and then

washed extensively with distilled water.

The fixed cell smears were immersed in Giemsa buffer (23mM Na2HP04 containing 8.5mM citric acid, pH 5.7) for 5 min. The slides were stained for 15 min with freshly diluted Giemsa stain (diluted 1:5 with the above buffer), rinsed with the same buffer, air dried, and examined under the microscope.

1.5 Preparation of membrane fractions and detergent extracts of human PBL

For the preparation of PBL membranes, isolated PBL (80 x 10^6 cells) were lysed by hypo-osmotic shock using H20 (20mls). An equal volume of 0.64M sucrose was then added and the cells were homogenised in a glass homogeniser with a motor-driven Teflon pestle (10 strokes, 1500r.p.m.). The homengenate was centrifuged (700g, 10 min, 4°C) and the supernatant was removed and recentrifuged (42,000g, 1h, 4°C ; Beckman ultracentrifuge). The resulting pellet, or P2 lymphocyte membrane fraction was resuspended in 0.32M sucrose (5ml) at 4°C.

Detergent extracts of human PBL were prepared by stirring human PBL (40 x 10^6/2ml) over ice for 30 min in BSS containing additionally 0.5% (v/v) Triton X-100. The extract was then centrifuged (42,000g, 1h, 4°C , Beckman ultracentrifuge) and the supernatant retained.

The PBL detergent extract and membrane fractions were subsequently used for [^{3}H] (-)- nicotine binding assays.

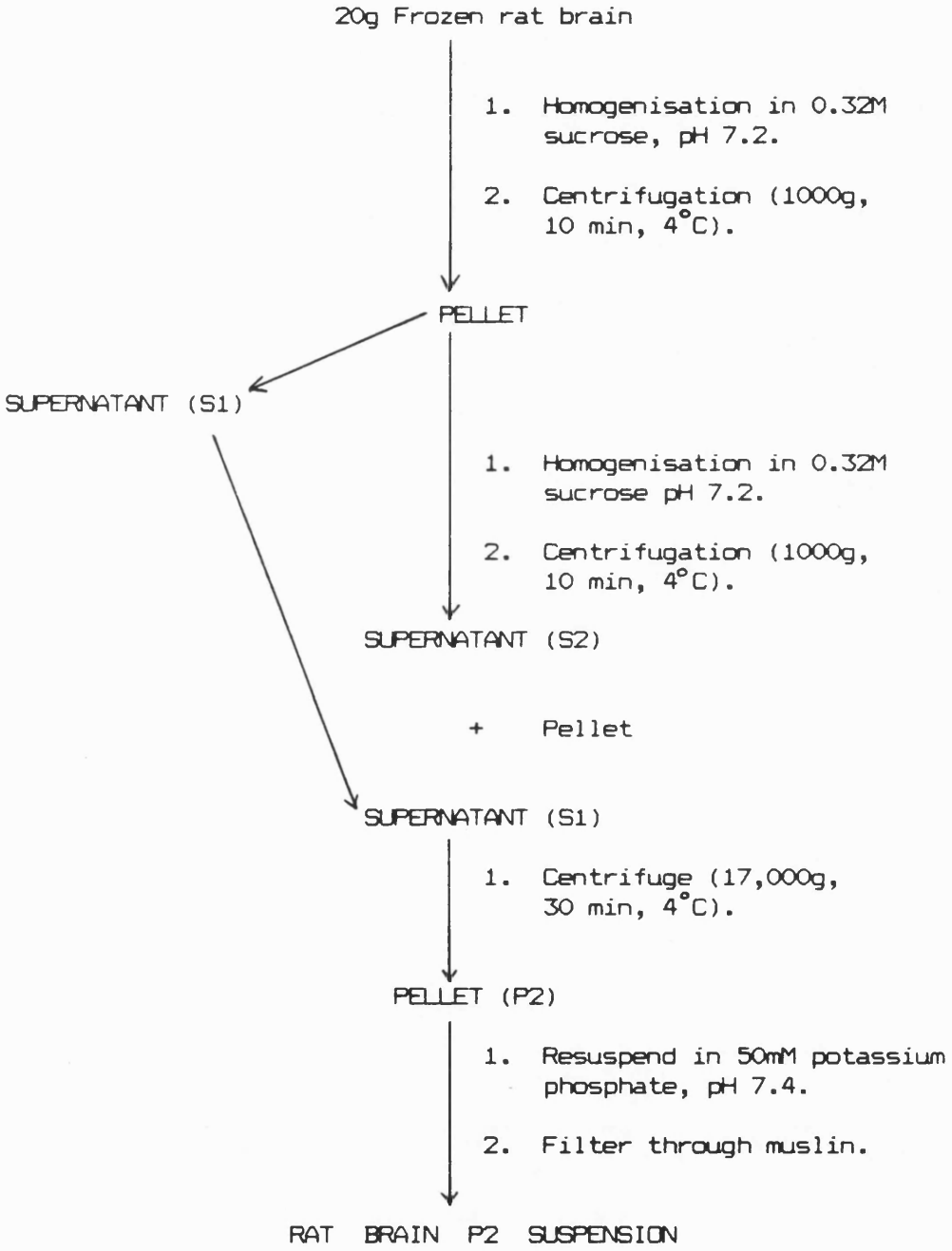
1.6 Preparation of P2 membrane fraction from rat brain.

The method used was essentially the same as that described by Whyte et al., (1985) and outlined in Figure 31 (page198).

Frozen rat brains from male Wistar rats (bodyweight 200g) were kindly provided by my colleagues. The brains had been quickly removed, chilled and stored at -20°C until required. All solutions contained 1mM EDTA, 1mM PMSF and 0.02% (w/v) sodium azide.

Frozen brains (20g) were added to chilled 0.32M sucrose, pH 7.2 (100ml) and left at 4°C for 30 min to thaw. The brain tissue was homogenised 0.32M sucrose (10 vol), using 2 x 6 strokes at 100 r.p.m. in a Teflon homogeniser. The resulting homogenate was centrifuged (1000g, 10 min, 4°C , MSE6L) and the supernatant (S1) decanted and retained on ice. The pellet was resuspended using a glass rod in 0.32M sucrose, pH 7.2 (5 vol) and centrifuged as before. The supernatant (S2) was removed, combined with the supernatant S1 and centrifuged (17,000g, 30 min, 4°C ; MSE6L). The resulting pellet, P2 , was resuspended to 2ml/g wet weight of original tissue in cold 50mM potassium phosphate buffer, pH 7.4 and filtered through a double thickness of muslin to remove any unresuspended material. The P2 suspension was stored at 4°C for up to one week.

Figure 31 General scheme of Rat brain P2 membrane preparation



2. Radioligand Binding Assays

Three cholinergic ligands were used in radioligand binding assays :- [^{125}I]-α-BGT, [^{3}H]-QNB and [^{3}H](-)-nicotine. A summary of the ligand binding assays used is given in Table 33.

2.1 [^{125}I]-α-BGT binding assays.

The basic procedure for [^{125}I]-α-BGT binding was the same for all tissue sources, Cell suspensions (1-50 x 10^{6} cells) or P2 rat membrane (0.5-2.0mg protein) were incubated with increasing concentrations of [^{125}I]-α-BGT (0-20nM), (specific activity 770 Ci/mmol) for 60 min at 22°C, in the presence and absence of 2.5 x 10^{-5} M α-BGT. The method of separation of free and bound radiolabel was either by centrifugation (P2 membrane and cells) or filtration (cells only). Sections D2.1.1 and D2.1.2 give the different protocols for each method. All determinations were made in triplicate.

2.1.1 Filtration assay

Cell suspensions (500 µl) were incubated with [^{125}I]-α-BGT (as above) in a total volume of 540 µl. The buffer used throughout was BSS (see Methods, Section D1.3). The mixture was filtered through pre-wetted DEAE-81 cellulose filters, GFB filters presoaked in BSS containing 2% (w/v) BSA or GFB filters presoaked in PEI (see Methods, Section A2.1) on a Millipore manifold. The filters were washed with BSS (4ml) and then counted for radioactivity.

2.1.2 Centrifugation assay

The method described is similar to that described by Rapier et al., (1985). Assays were carried out in Eppendorf tubes which had been coated with PBS containing 1% (w/v) casein for 1h and washed twice with PBS prior to use. Cell suspensions or P2 membrane (500 µl) were incubated in a final assay volume of 540 µl. The buffer used for P2 membranes was 500mM potassium phosphate buffer containing 1mM EDTA, 0.5mM PMSF, 0.02% (w/v) sodium azide, pH 7.4 and for cell suspensions, BSS. Bound radioligand was separated by centrifugation (1 min, high speed, MSE microfuge) and the tissues were washed three times (total volume 4.5ml) with ice cold buffer; which was PBS and BSS for P2 membranes and cells respectively. The pellets were then counted for radioactivity.

2.2 [^{3}H]-QNB binding assays.

The binding of [^{3}H]-QNB to mouse thymocytes, human PBL and erythrocytes was carried out by using a similar protocol to that described in Section D2.1.1. The differences are given here.

For saturation studies, the lymphocyte suspensions were incubated (1h, 22°C) with increasing concentrations of [^{3}H]-QNB (5-200nM, specific activity 46Ci/mmol) in the presence and absence of 10^{-3}M atropine. The specific binding of [^{3}H]-QNB was defined as the amount of [^{3}H]-QNB bound in the absence of competing ligand minus the amount bound in the presence of atropine. Competition studies were usually carried out at 20nM [^{3}H]-QNB.

2.3 [^{3}H] (-)-Nicotine binding assays.

The binding of [^{3}H](-)-nicotine to rat brain P2 membranes, mouse thymocytes and human PBL was performed by a method described by Rapier et al., (1985).

Lymphocytes (240 µl, 0.5-2 x 10^6 cells, or lymphocyte membranes) or P2 membranes (240 µl, 0.5-2.0mg protein/ml) were incubated in a final volume of 300 µl with [^{3}H](-)-nicotine (usually 5-100nM, specific activity 60-85 Ci/mmol) for 30 min at 22°C and 1h at 4°C in the presence and absence of 1mM nicotine (-) ditartrate. After incubation, samples were diluted with wash buffer (see below) (2ml at 4°C) and filtered under vacuum on GFC filters (P2 membranes) or GFB filters (lymphocytes) presoaked in water containing 0.03% (v/v) PEI (Methods, Section A2.1b) for 1h prior to use. The filters were washed twice with wash buffer or distilled water (total volume 4ml). The procedure was carried out quickly (~30s) from dilution to last rinse. The filters were transferred to scintillation vials and Optiphase scintillation fluid added (5ml), left overnight at 4°C and counted for tritium. Counting efficiency was 36%.

For rat brain P2 membranes, incubation and washing buffer was Hepes buffer (20mM Hepes-4-(2-hydroxyethyl)-1-piperazine-ethane sulphonic acid, 118mM NaCl, 4.8mM KCl, 1.2mM $MgSO_4$. $7H_2O$, 2.5mM $CaCl_2$, pH adjusted to 10.5 with 10M NaOH). For lymphocyte studies the incubation and washing buffer was BSS.

2.4 Binding kinetics.

The time course for nicotine binding was determined by

incubating a fixed concentration of [^{3}H]-(-) nicotine (20nM) and human PBL (1 x 10^6 cells) for varying amounts of time (0-60 min), at 23°C. Incubations were terminated at successive times by filtration as described in the previous section.

The time course for nicotine dissociation was followed by first incubating [^{3}H](-)-nicotine (20nM) and human PBL (1 x 10^6 cells) until equilibrium was achieved, (60 min at 23°C). This incubation was followed by the addition of excess of unlabelled nicotine (-) ditartrate (10mM), and rapid filtration at successive times (0-10 min). For each time point in all the kinetic studies, total binding and non-specific binding were determined in triplicate.

2.5 Competition studies using test drugs and anti-(AChR) monoclonal and polyclonal antibodies.

Competition assays using test drugs were conducted by preincubating the tissue sample (rat brain P2 membrane or human PBL) with varying concentrations of test drug (30µl) for 5 min before the addition of radioligand. Inhibition assays involving the use of 2-methyl piperidine and pyrrolidine were carried out by using siliconised glass tubes.

Similarly, rat brain P2 membranes or human PBL were preincubated with either polyclonal anti-(AChR) antisera T , F1 or F2 (30 µl) (see Results, Section C.1) or anti-(AChR) Mab ascites fluid (20 µl) (see Results, Section C2.1) for 15 min prior to the addition of [^{3}H] (-)-nicotine. Rabbit anti-(BSA) antiserum and mouse anti-(BSA) Mab ascites fluid were used as controls.

RESULTS

1. Isolation and characterisation of leucocyte populations

1.1 Separation and yield of human PBL and granulocytes

1.2 Giemsa staining of human PBL and granulocyte cell fractions

2. Cholinergic binding sites on leucocyte and membrane fractions

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2.1.2b [^{125}I]-α-BGT binding to mouse thymocytes

2.1.2c [^{125}I]-α-BGT binding to human PBL

2.2 Binding of [^{3}H]-QNB to lymphocyte preparations

2.2.1 [^{3}H]-QNB binding to mouse thymocytes

2.2.2 [^{3}H]-QNB binding to mouse erythrocytes

2.2.3 Binding of [^{3}H]-QNB to human PBL

2.2.3a Relationship of radioligand binding and cell number

2.2.3b Saturation curves and Scatchard analysis

2.2.3 Inhibition of [^{3}H]-QNB binding to human PBL by atropine

2.2.4 Binding of [^{3}H]-QNB to human erythrocytes

2.3 [^{3}H]-nicotine binding studies

2.3.1 [^{3}H] (-) - nicotine binding to mouse thymocytes

2.3.2 [^{3}H] (-) - nicotine binding to human PBL

2.3.2a Linearity of binding with cell number

2.3.2b Saturation curves and Scatchard analysis

2.3.2c Determination of the association rate constant for [^{3}H] (-) - nicotine binding to human PBL

2.3.2d Determination of the dissociation rate constant for [^{3}H] (-) - nicotine binding to human PBL

2.3.3 [^{3}H] (-) - nicotine binding to human granulocytes

2.3.4 [^{3}H] (-) - nicotine binding to human erythrocytes

2.4 Competition studies

2.4.1 Steroselectivity of [^{3}H] (-) - nicotine binding

2.4.2 Pharmacological specificity of [^{3}H] (-) - nicotine binding to human PBL

2.4.3 Inhibition studies using anti-(nAChR) monoclonal and polyclonal antibodies

2.5 Effect of heat on the binding of [^{3}H] (-) - nicotine to human PBL

2.5.1 Heat inactivation of [^{3}H] (-) - nicotine binding to human PBL

2.5.2 Comparison of incubation temperatures on [^{3}H] (-) - nicotine binding

2.6 Binding of [^{3}H] (-) - nicotine to subcellular fractions of human PBL

2.6.1 [^{3}H] (-) - nicotine binding to detergent extract of lymphocytes

2.6.2 [^{3}H] (-) - nicotine binding to lysed lymphocytes

1. Isolation and characterisation of leucocyte populations

The aim of the work described in this chapter was to investigate the presence of cholinergic binding sites on lymphocyte fractions. Suspensions of various cells were prepared and used in radioligand binding assays. The P2 membrane fraction of rat brain was used as a well characterised tissue source for establishing methodology and for comparative studies.

1.1 Separation of human PBL and granulocytes

PBL were isolated from whole heparinsed blood, leucopheresis and buffy coat samples by Ficoll-Hypaque density centrifugation as described in the Methods Section D1.

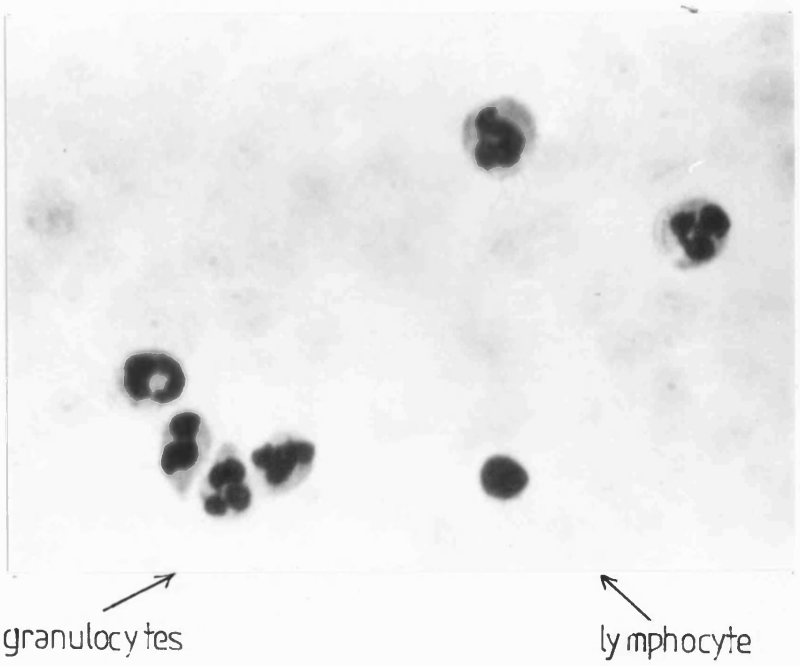
The yield of PBL from freshly drawn blood from six normal healthy volunteers was $21.2 \pm 1.0 \times 10^6$ cells per 20ml blood sample (mean $\pm$ S.E.). The yield of lymphocytes from buffy coat samples (50 ml) was $2.38 \pm 0.76 \times 10^8$ cells (mean $\pm$ S.E.; n = 15). Leucopheresis samples were obtained from two patients with MG and the yield of PBL obtained from 50ml samples was 6.25×10^8 and 3.3×10^9 cells. Patient 1 and patient 2 had anti-(AChR) titres of 0.28×10^{-6} M and 9.0×10^{-9} M respectively.

Granulocytes were prepared from buffy coat blood samples only (Methods, Section D1) giving yields of $1.02 \pm 0.54 \times 10^7$ cells (mean $\pm$ S.E.; n = 3).

1.2 Giemsa staining of human PBL and granulocytes

The PBL and granulocyte populations obtained from one buffy coat sample were examined by using Giemsa stain on fixed cell smears.

FIGURE 32 Giemsa staining of human PBL and granulocytes



Human PBL and granulocytes were stained using Giemsa stain (Methods D14) and classified according to the morphology of their nuclei.

Cells were classified according to the morphology of their nuclei. Examples of the different cell types are shown in Figures 32 (page207) Cell counts were obtained by choosing six non-overlapping fields of view and expressing the number of cell types observed as a % of the total number of cells counted. The results are shown in the Table 32 below. The preparation of PBL was relatively pure with approximately 15% contamination with other cell types. The granulocyte preparation, similarly, had little contamination from lymphocytes and monocytes.

Table 32 Characterisation of Cell populations isolated by Ficoll-Hypaque Density centrifugation

Ficoll-Hypaque Fraction	Granulocytes	Lymphocytes	Monocytes
Top Fraction	2.2%	85%	12%
Bottom Fraction	96%	2%	0.3%

2. Cholinergic binding sites on leucocyte and membrane fractions

2.1 Binding of [^{125}I]-α-BGT to lymphocyte preparations

Specific binding of [^{125}I]-α-BGT to mouse thymocytes and human PBL from healthy donors and MG patients was assessed as the difference in the binding of [^{125}I]-α-BGT in the presence and absence of 10^{-6} M unlabelled α-BGT. The separation of free from bound radioactivity was carried out by using filter assays and centrifugation assays (Methods, Section D2.1). A number of modifications were made to the methods before successful results were obtained.

2.1.1 Filter assays

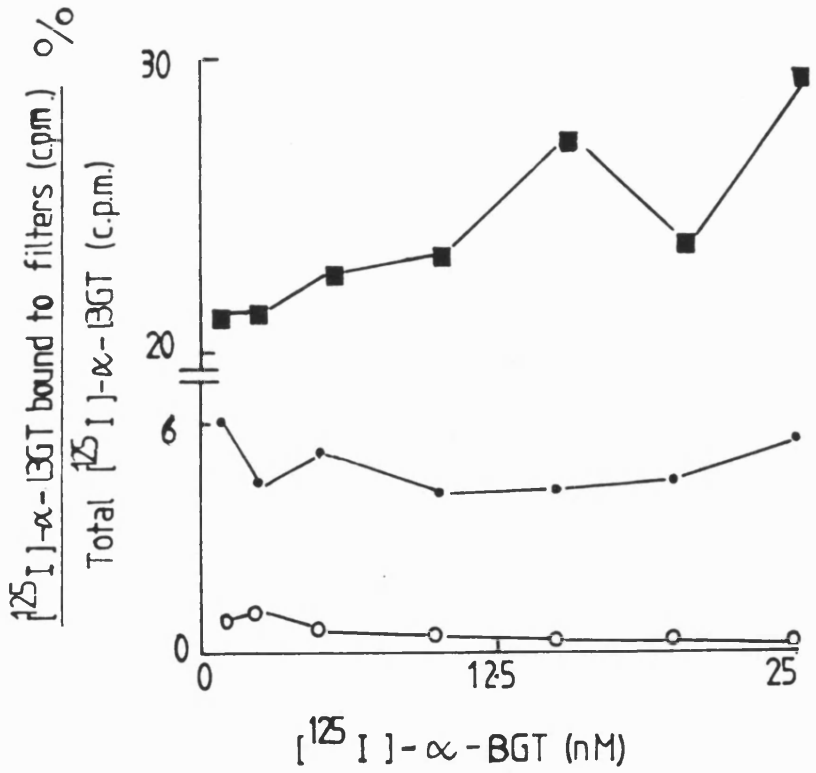
DEAE-filters were used routinely in the assay of purified AChR (Methods, Section A2.2) and this method was used in initial experiments to detect [^{125}I]-α-BGT binding to lymphocyte preparations.

Initial experiments were carried out by using BSS (Methods, D1.3) as assay and washing buffer and it became apparent that binding of free [^{125}I]-α-BGT to DEAE filters represented a significant source of error, especially at higher concentrations of [^{125}I]-α-BGT (see Figure 33, page 209). Such errors may be important when trying to detect an unknown but probably small population of binding sites.

Several experiments were carried out to reduce the non-specific binding of [^{125}I]-α-BGT to filters by soaking filters in solutions of 2% (w/v) BSA, 1% (w/v) casein and 0.1% (w/v) poly-L-lysine. The inclusion of 2% (w/v) BSA in the washing buffer and presoaking filters in this buffer was effective in reducing non-specific binding to DEAE filters (see Figure 33, page 209). In later experiments (n = 3) PEI-treated GFB filters were used, as described by Bruns et al., (1983). This method further reduced non-specific binding to 0.6% of the total counts added (see Figure 33, page 209).

The apparent kd values from equilibrium studies of [^{125}I]-α-BGT binding to purified AChR are in the order of 2×10^{-9} M (Lukasiewicz et al., 1978) hence [^{125}I]-α-BGT binding to lymphocyte preparations was studied over a concentration range

Figure 33 Non-specific binding of [^{125}I]-α-BGT in the filtration assay



Increasing concentrations of [^{125}I]-α-BGT were filtered through DEAE-81 filters presoaked and washed with B.S.S. (-■-) and B.S.S. containing 2% (w/v) BSA (-•-) or GFB filters soaked with PEI (-○-). The radioactivity retained by the filters was expressed as a % of the total radioactivity applied to the filter.

of 0-25nM. In three experiments this range was extended to 60nM in order to detect any lower affinity binding sites. No specific binding of [^{125}I]-α-BGT to lymphocytes was detected using any of the modifications described. Table 33 (page 211 Section D2) gives a summary of the approaches used.

The effects of varying the number of cells used for each determination was also investigated at a fixed concentration of [^{125}I]-α-BGT, usually 20nM. There was an obvious limitation in the number of cells that could be used, especially for determinations made in triplicate and especially for human lymphocytes where the number of lymphocytes obtained was $\sim$ 2 x 10^8 per sample (see Results, Section D1). The usual cell number used per assay was increased from 2 x 10^6 cells to 10 x 10^6 cells. However, no specific binding was observed at this higher cell number which incidently led to slow filtration because of clogging of the filters. An alternative assay method, that of centrifugation was accordingly investigated.

2.1.2 Centrifugation assay

The binding of [^{125}I]-α-BGT to rat brain P2 membranes by centrifugation is a well established method in the laboratory (see Wonnacott et al., 1986) and the binding has been well characterised (see Schmidt et al., 1980). This tissue source was therefore used initially to establish the efficacy of the centrifugation assay in my hands.

2.1.2a [^{125}I]-α-BGT binding to rat brain P2 membranes

The binding of [^{125}I]-α-BGT binding to rat brain P2 membrane

Table 33 Summary of ligand binding assay methods

Radioligand	Competing ligand for measuring Non-specific binding	Incubation Time, Temperature	Method of Separation	Tissue	Type of filter
[^{125}I] -α-BGT	α-BGT	1h, 22°C	Filtration	Mouse thymocytes	DEAE-81/BSA
				Human PBL	GFB/BSA GFB/PE1
			Centrifugation	Rat P2 membrane	
				Mouse thymocytes	-
				[a] Human PBL	
[^{3}H] (-)-nicotine	(-)-nicotine ditartrate	30min, 22°C + 1h, 4°C	Filtration	Rat P2 Membrane	GFC/PE1
				Mouse thymocytes	GFB/PE1
				Human PBL	"
[^{3}H] -QNB	atropine	1h, 22°C	Filtration	Mouse thymocytes erythrocytes	GFB/PE1
				Human PBL erythrocytes	

[a] The human PBL samples were from normal controls and patients with MG.

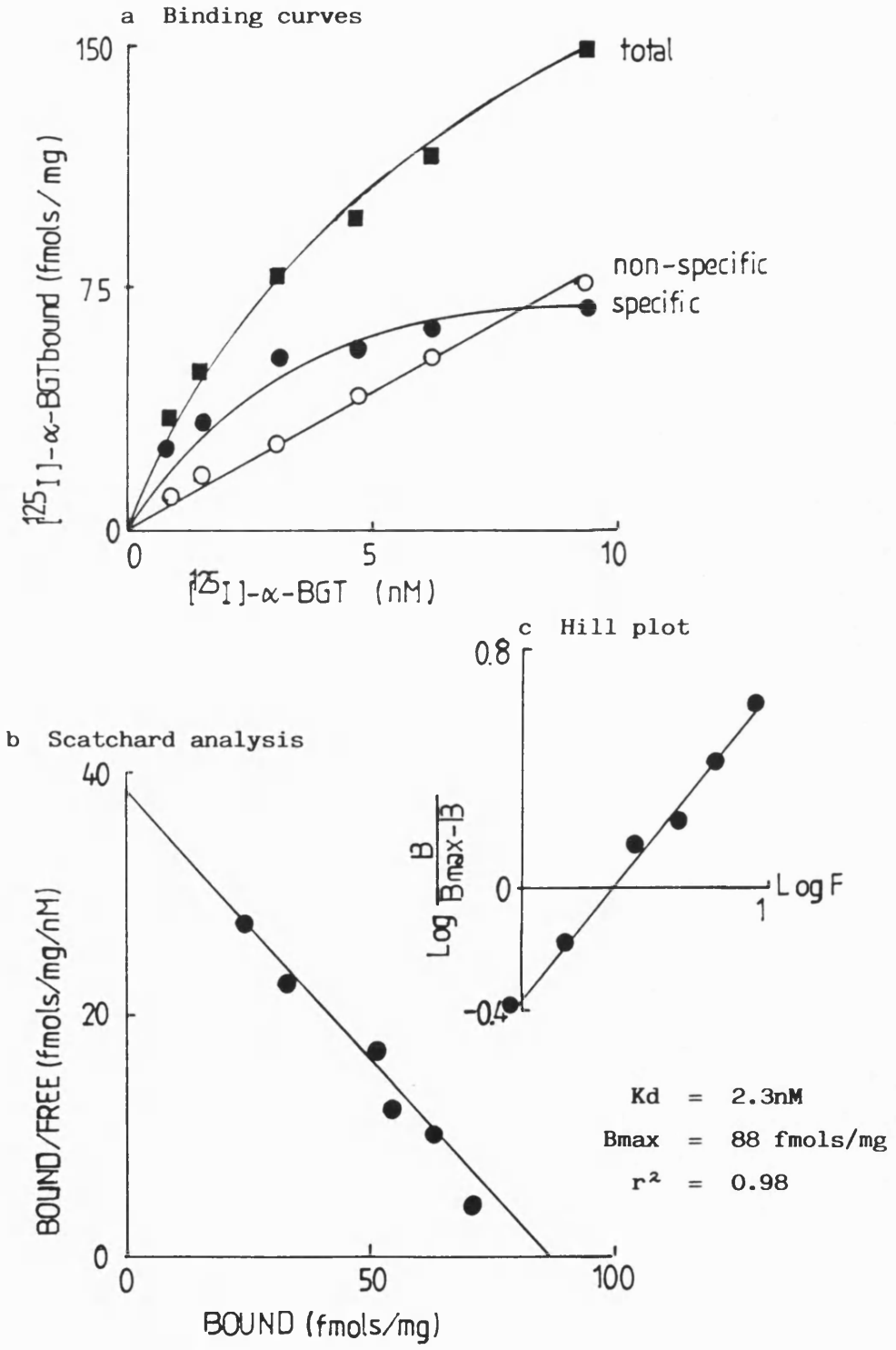
was saturable (Figure 34 a, page 213) with linear non-specific binding (r^2 = 0.98). Scatchard analysis (Figure 34 b, page 213) gave an apparent Kd of 2.3 nM and Bmax 8 8 fmols/mg protein (r^2 = 0.98). The Hill plot (Figure 34 c, page 213) gave a Hill coefficient Nh = 1.06 indicating a single class of binding sites.

2.1.2b [^{125}I]-α-BGT binding to mouse thymocytes

Cell numbers greater than those used for filtration could be used in centrifugation assays and in experiments using mouse thymocytes the cell number used per assay tube was increased to 20 x 10^6 and in one experiment to 50 x 10^6. The non-specific binding of [^{125}I]-α-BGT to lymphocytes represented approximately 0.09% of the total counts added and this constitutes a 6 and 50 fold improvement on the GFB/PEI and DEAE/BSA assays respectively (see Table 34 , page 213). High speed centrifugation tended to clump the cells making them difficult to wash. Hence, centrifugation at low speed for ~ 30s was used. The addition of 2% (v/v) FCS to the washing buffer also helped in resuspending the cells during the wash steps.

The binding of [^{125}I]-α-BGT to mouse thymocytes was still however, found to be variable and in only one experiment out of five was saturable binding observed (Figure 35 , page 215). This experiment used 50 x 10^6 cells per assay tube. One fmole of [^{125}I]-α-BGT bound corresponded to approximately 1300 cpm. Non-specific binding was linear and Scatchard analysis (Figure 35a , page 215) gave an apparent Kd of 6.3nM and

Figure 34 [^{125}I]-α-BGT binding to Rat brain P2 membranes



Bmax 3.4 fmols/ 10^6 cells (r^2 = 0.87), equivalent to 2000sites per cell, assuming all cells have specific α-toxin binding sites. The Hill plot gave a Hill coefficient Nh = 1.09 (r^2 = 0.93).

Table 34 Comparison of Non-specific binding and reproducibility [^{125}I]-α-BGT binding assays in the presence of tissue

Assay Procedure	Non-specific binding as a % of Total counts added
DEAE Assay	4.7 ± 0.436%
GFB/PEI	0.61 ± 0.073%
Centrifugation	0.088 ± 0.009%

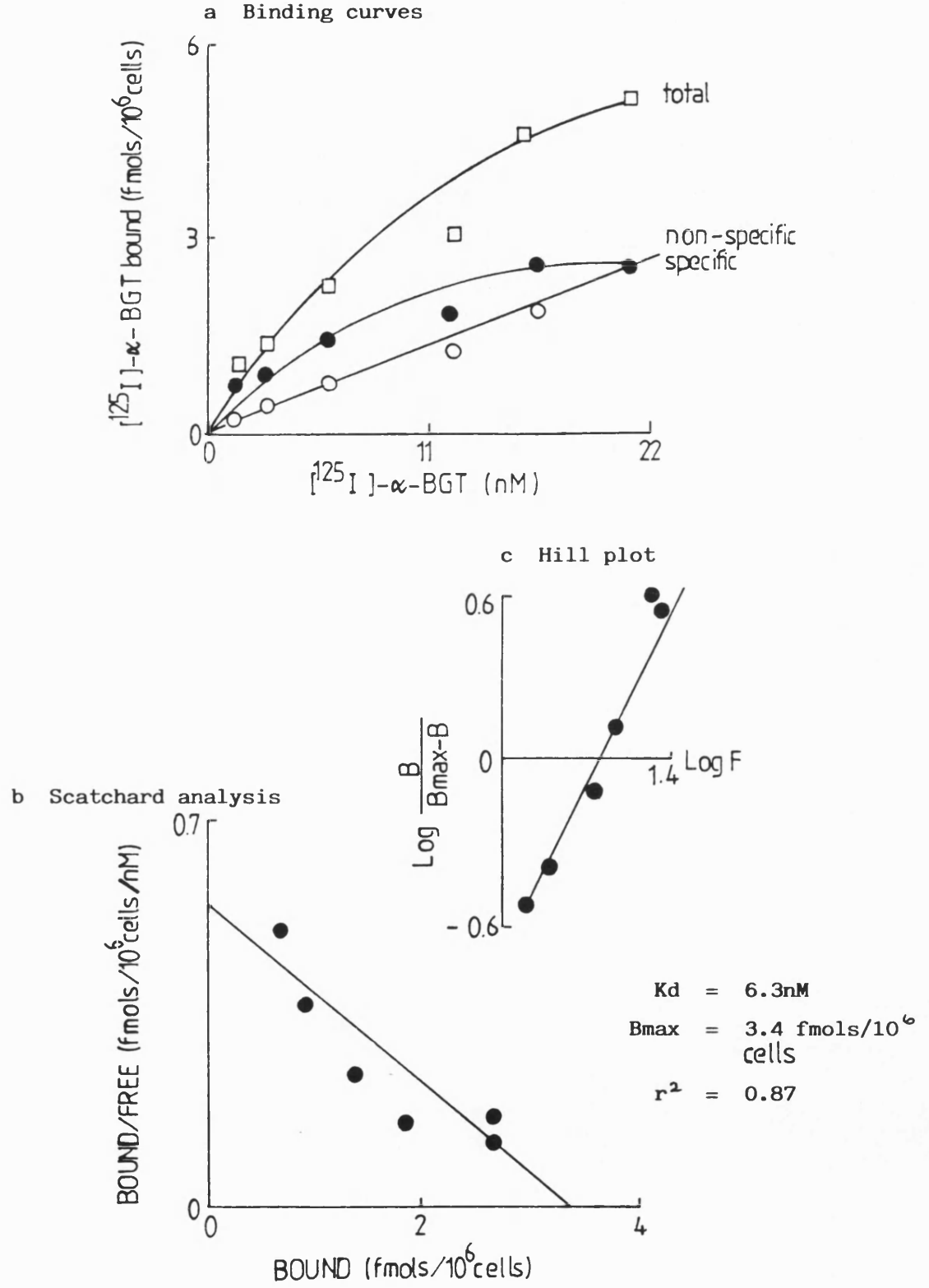
Experiments were carried out by using the same number of thymocytes (10 x 10^6) and are the mean ± S.E. of 10 determinations at 16nM [^{125}I]-α-BGT.

2.1.2c [^{125}I]-α-BGT binding to human PBL

Because of the limited number of human PBLs available from each blood sample, it was impossible to use cell densities as high as those in Results, Section D2.1.2b. Hence, PBL were used at 15 x 10^6 cells per assay tube. If binding sites were present at the same density as shown in one experiment with mouse thymocytes (Results, Section D2.1.2b) one would expect specific counts of approximately 4000. However, no specific binding of [^{125}I]-α-BGT was observed to normal PBL (n = 9) or PBL from two myasthenic patients using these conditions.

Because of the poor reproducibility of [^{125}I]-α-BGT binding to

Figure 35 [^{125}I]-α-BGT binding to mouse thymocytes



mouse thymocytes and apparent lack of specific [^{125}I]-α-BGT binding sites on human lymphocytes, these binding studies were discontinued. In order to verify the binding methods used, the binding of [^{3}H]-QNB to lymphocyte populations was examined, as this system has been well documented in the literature for the detection of mAChR (Gordon et al., 1978; Lopker et al., 1980; Strom et al., 1981; Zalcman et al., 1981; Atweh et al., 1984; Adem et al., 1986a,b).

The binding of [^{3}H]-QNB to lymphocyte preparations was carried out as described in the Methods, Section D2.2.

2.2 Binding of [^{3}H]-QNB to lymphocytes

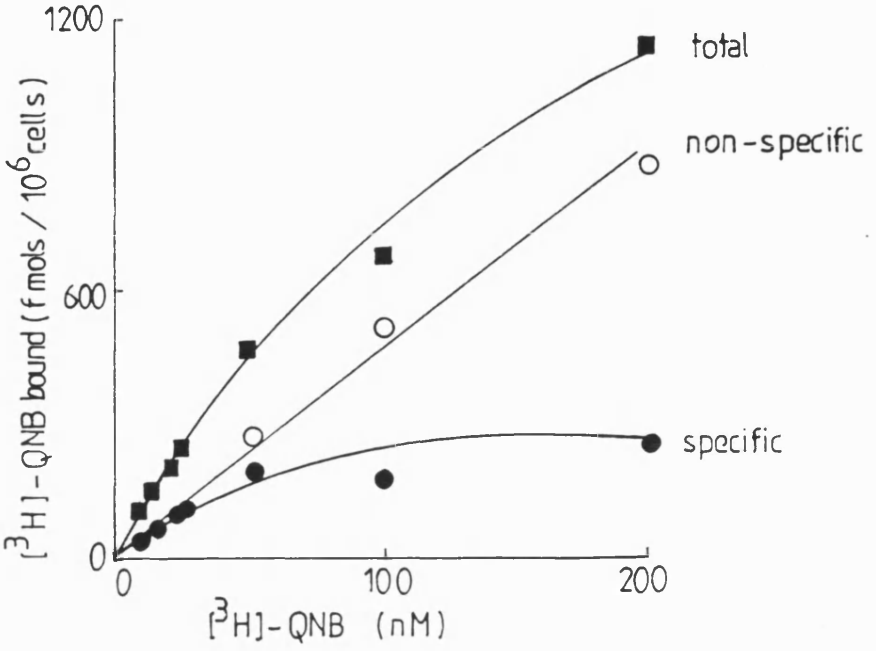
The binding of [^{3}H]-QNB was assessed over the concentration range 0-200nM. Specific binding was determined as the difference between [^{3}H]-QNB binding in the absence and presence of the antagonist, atropine sulphate. The non-specific binding of [^{3}H]-QNB to GFB/PEI filters was <1% of the total radioactivity added in the absence of tissue using 6ml wash volumes.

2.2.1 [^{3}H]-QNB binding to mouse thymocytes

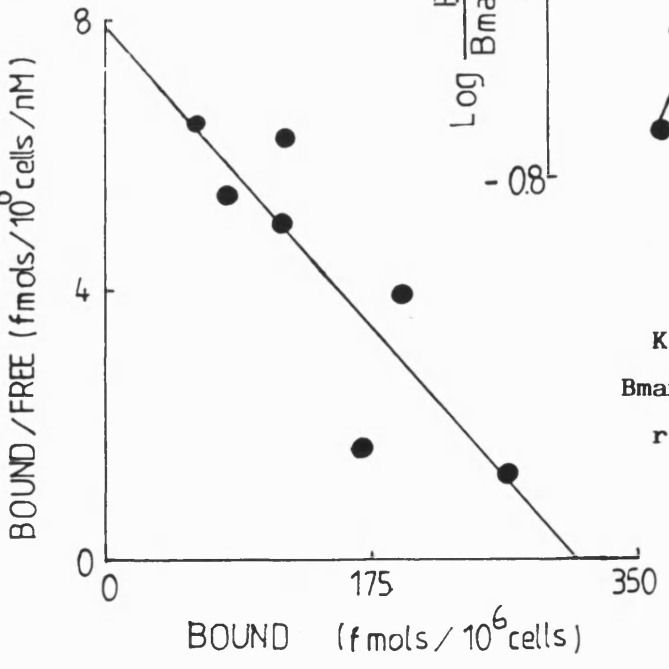
The specific binding of [^{3}H]-QNB to mouse thymocytes was saturable, with linear non-specific binding (Figure 36 a, page 217). Scatchard analysis of the data (Figure 36 b, page 217) gave an apparent Kd of 39nM and Bmax 314 fmols/10^{6} cells, which is approximately equivalent to 2 x 10^{5} sites/cell. The Hill coefficient Nh = 1.04 (r^{2} = 0.94) indicated a single class of binding sites (Figure 36 c, page 217).

Figure 36 [^{3}H]-QNB binding to mouse thymocytes

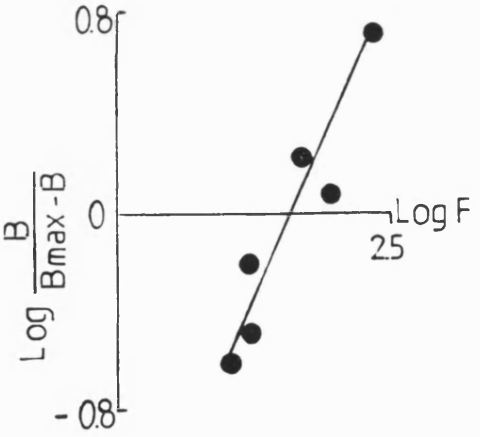
a Binding curves



b Scatchard analysis



c Hill Plot



Kd = 39nM

Bmax = 314 fmols/10^6 cells

r^2 = 0.86

2.2.2 [^{3}H]-QNB binding to mouse erythrocytes

The contamination of the thymocyte cell suspension by erythrocytes in this experiment was 5%. Human erythrocytes are known to possess specific [^{3}H]-QNB binding (Aronstam et al., 1977). To control for possible binding to murine erythrocytes, red cells were prepared (from blood collected by cardiac puncture) and incubated with [^{3}H]-QNB under conditions identical to those used for the thymocyte population.

Specific [^{3}H]-QNB binding sites were detectable on mouse erythrocytes (Figure 37 a, page 219), however, the observed binding affinity was much lower than that for thymocytes, with Scatchard analysis (Figure 37 b , page 219) giving a Kd of approximately 250nM and a Bmax value of 3.9 fmols/10^6cells, (equivalent to 2300 binding sites/cell, r^2 = 0.82). Hence a 5% erythrocyte contamination contributed <0.2 fmols [^{3}H]-QNB binding and was thus considered insignificant.

2.2.3 Binding of [^{3}H]-QNB to human PBL

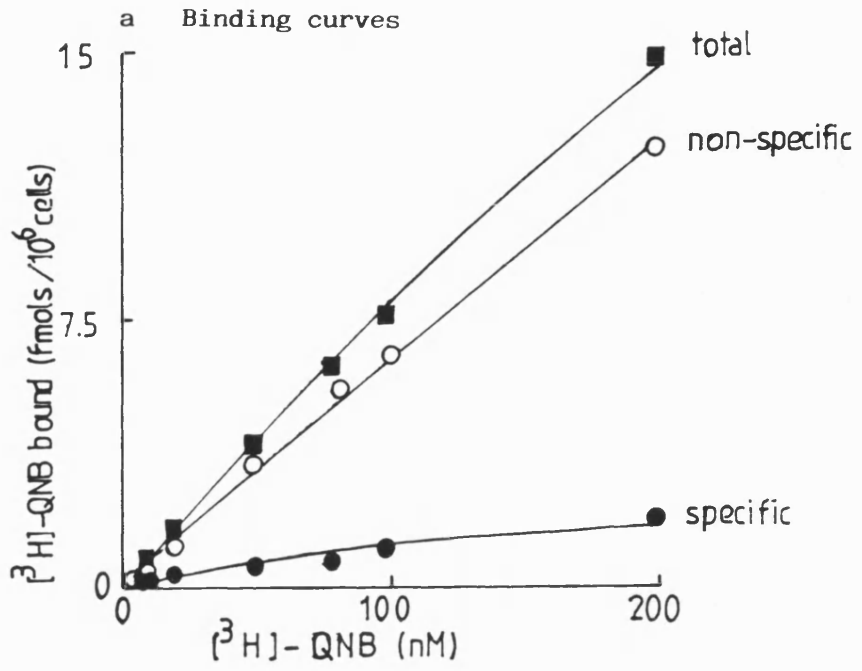
2.2.3a Relationship of radioligand binding and cell number

The binding of [^{3}H]-QNB (40nM) to normal human PBL was shown to be linear over the range 0-1 x 10^6 cells/assay tube (Figure 38). Subsequent binding assays were carried out using 1 x 10^6 cells.

2.2.3b Saturation curves and Scatchard analysis

The binding of [^{3}H]-QNB to human PBL was highly reproducible over the concentration range 5-200nM. A typical binding curve is shown in Figure 39 (page 221). Scatchard analysis of the

Figure 37 [^{3}H]-QNB binding to mouse erythrocytes



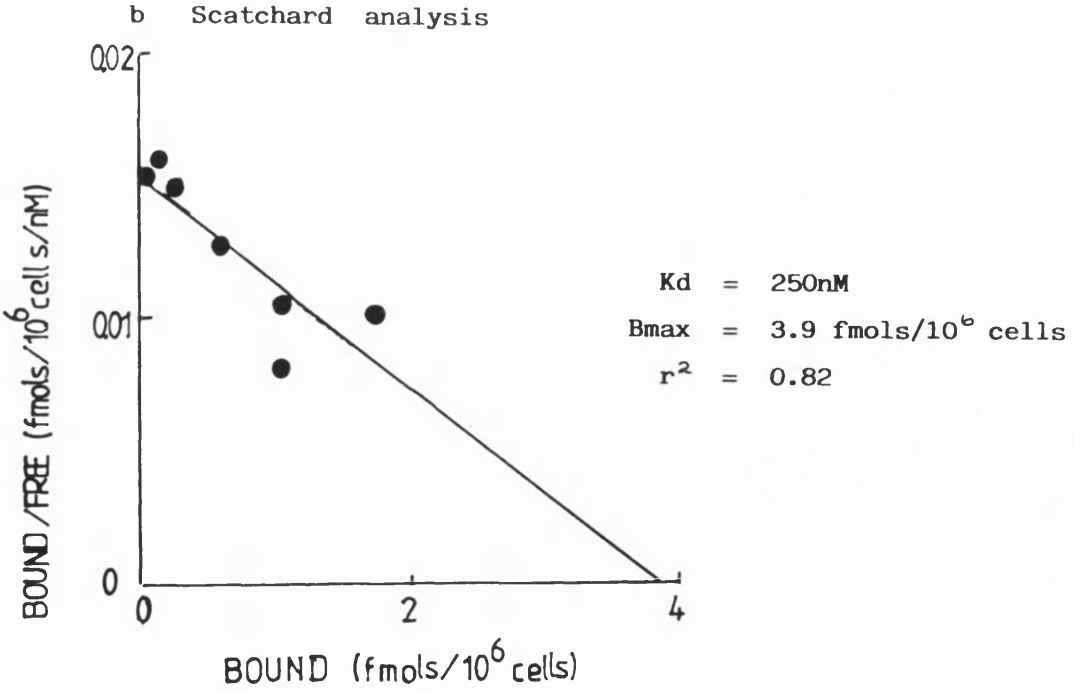
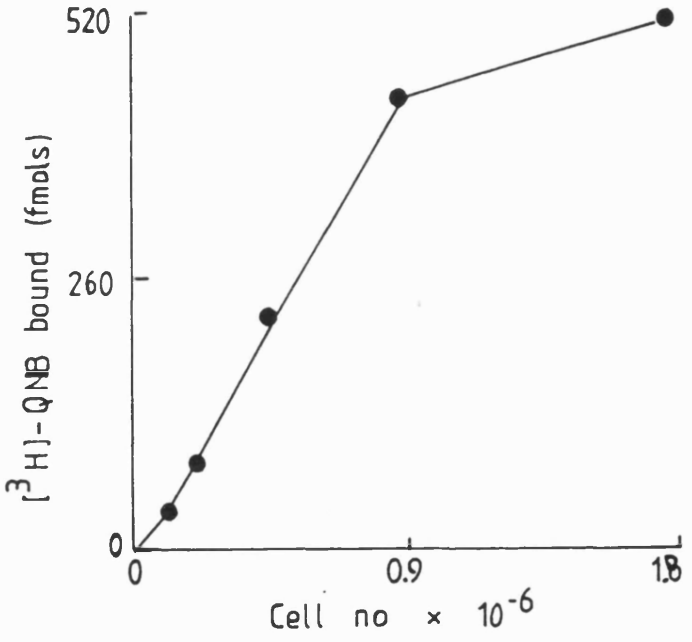


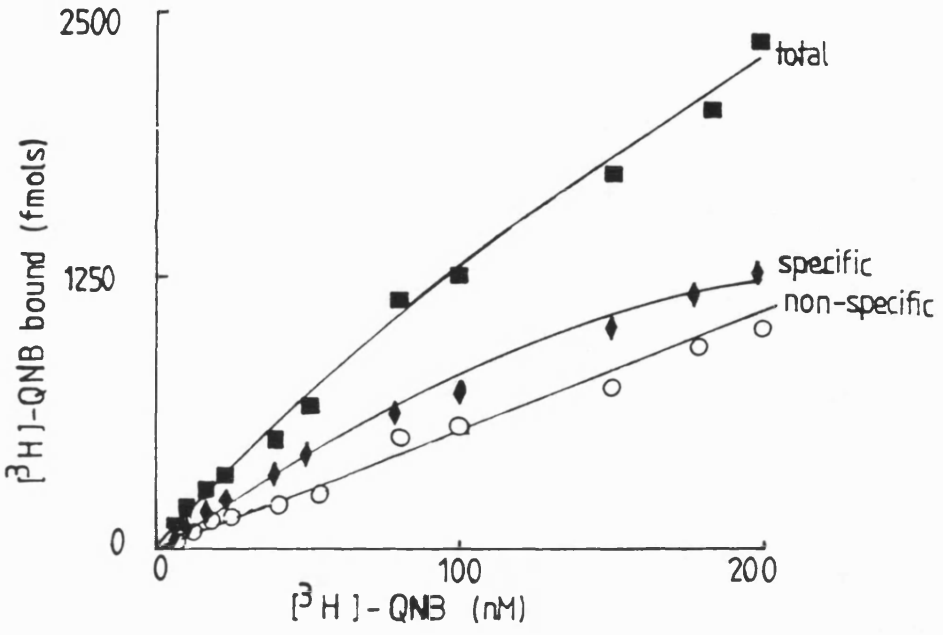
Figure 38 Specific [^{3}H]-QNB binding as a function of cell number



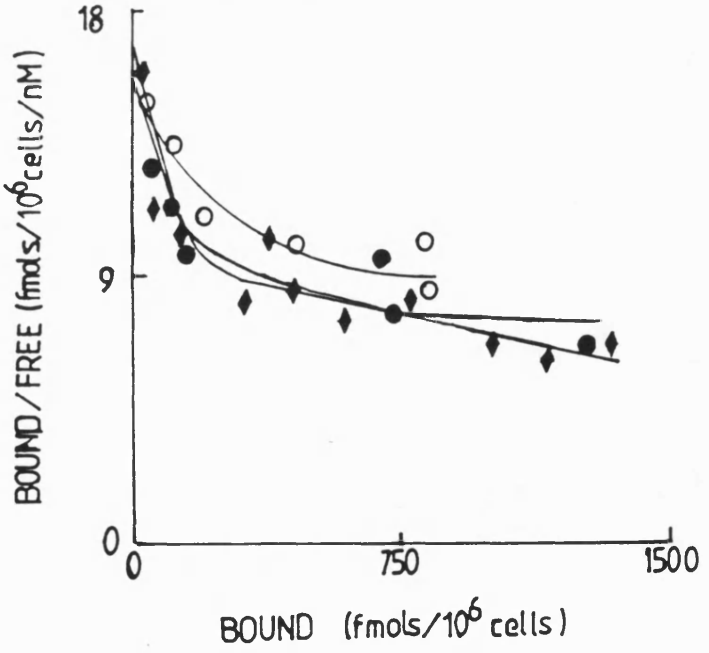
Results of triplicate determination of specific [^{3}H]-QNB binding at 40nM.

Figure 39 [^{3}H]-QNB binding to human PBL

a Binding Curve



b Scatchard analysis



data from experiments using isolated PBL from three different blood samples is shown in Figure 39 . Curvilinear Scatchard plots were obtained for the three samples studied, suggesting either cooperativty or heterogeneity of binding sites. Further analysis of the data using a computer program ANOVA gave a high affinity binding site with a Kd of 69nM and Bmax of 510 fmols/10^6 cells and a low affinity site of Kd 233nM and 1750 fmols/10^6 cells (F = 138.8 at 4 and 15 Degrees Freedom).

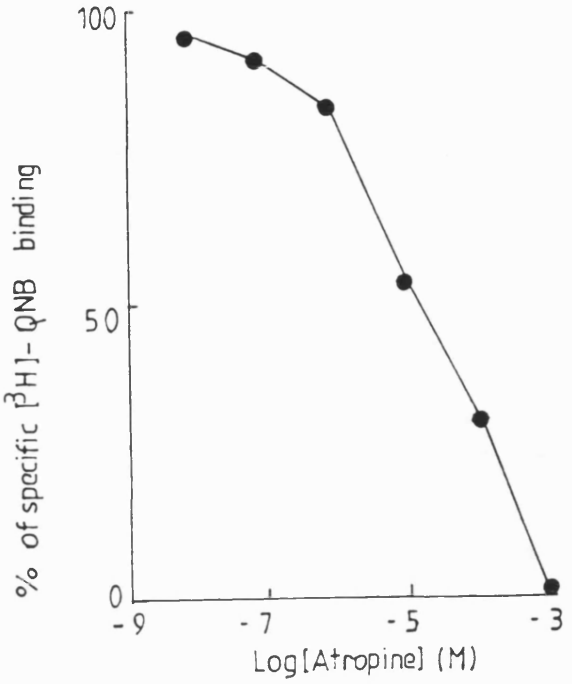
2.2.3c Inhibition of [^{3}H]-QNB binding to human PBL by atropine
An inhibition curve of [^{3}H]-QNB binding to human PBL by varying concentrations of atropine is shown in Figure 40 . The data were analysed to yield the Hill plot (Figure 41) to derive the IC50 value (concentration of drug that inhibits specific binding by 50%) of 2.5 x 10^{-5} M.

2.2.4 Binding of [^{3}H]-QNB to human erythrocytes
Erythrocytes were prepared from buffy coat samples depleted of white cells by centrifugation and aspiration of the supernatant. Contamination of the erythrocytes by white blood cells, as estimated by the method described in Section D1.1 was 0.5%.

There was no specific binding of [^{3}H]-QNB over the concentration range 0-200nM.

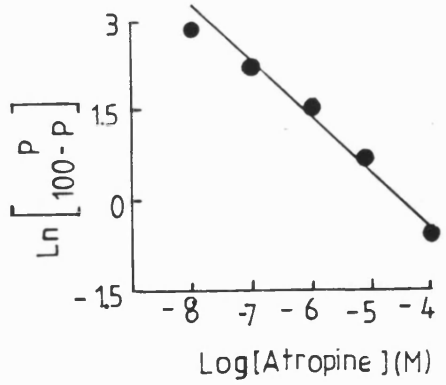
2.3 [^{3}H] (-)- nicotine binding studies
It has been shown that not all nAChRs bind [^{125}I]-α-BGT (see Introduction, Section 3.6). In these instances the radioligand

Figure 40 Inhibition of [^{3}H]-QNB binding to human PBL by atropine



Varying concentrations of atropine were preincubated with 8×10^5 human PBL, followed by incubation with [^{3}H]-QNB (200nM). Each point is the mean of triplicate determinations.

Figure 41 Linear transformation of above data to yield the HILL PLOT



P represents the percentage bound at each concentration of atropine.

[^{3}H] (-)- nicotine has proved a useful and specific probe for detecting the non α-BGT binding subclasses of nAChRs. Hence, [^{3}H](-)- nicotine was used as an alternative probe for nicotinic cholinergic receptors on lymphocyte preparations.

The binding of [^{3}H] (-)- nicotine to lymphocyte populations was initially studied by using mouse thymocytes because they were easier to obtain in large numbers than human PBL.

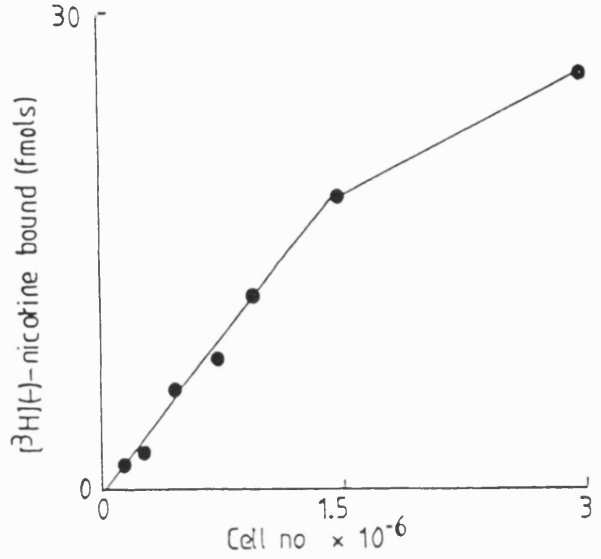
2.3.1 [^{3}H] (-)- nicotine binding to mouse thymocytes

The first experiments carried out determined the binding of [^{3}H] (-)- nicotine to increasing numbers of mouse thymocytes. The specific binding in the presence of 10^{-3}M nicotine (-) ditartrate was found to be linear over the range 0-1.5 x 10^6 cells (Figure 42a) and subsequently 1 x 10^6 cells were used for a saturation binding study (Figure 43).

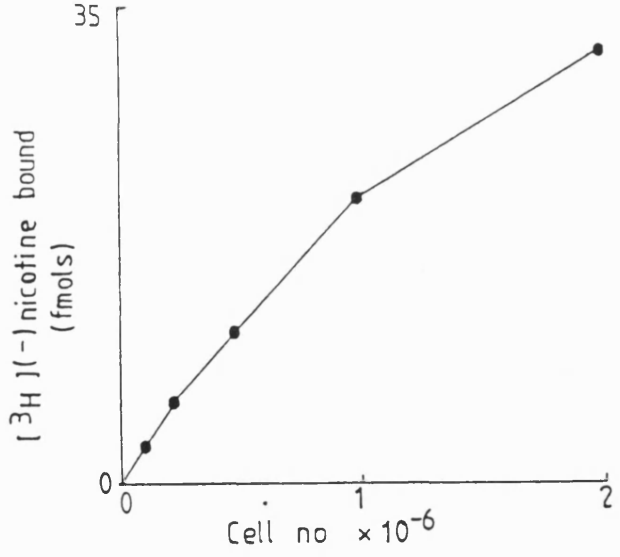
The binding of [^{3}H] (-)- nicotine to mouse thymocytes was studied over the concentration range 0-100nM (Figure 43). Linear non-specific was observed. Binding was not saturable at 100nM, however it was not possible to use higher concentrations of the radioligand because of its high cost. However, Scatchard analysis of the binding data shown in Figure 43 a yielded a straight line (r^2 = 0.84) giving a Kd of approximately 136nM and a Bmax value of 56 fmoles/10^6 cells (34000 binding sites/cell) (Figure 43 b). The Hill coefficient Nh = 1.04 indicated a single class of binding sites (r^2 = 0.96) (Figure 43 c).

Figure 42 Specific [^{3}H]-(-)-nicotine binding to lymphocyte populations as a function of cell number

a Mouse thymocytes

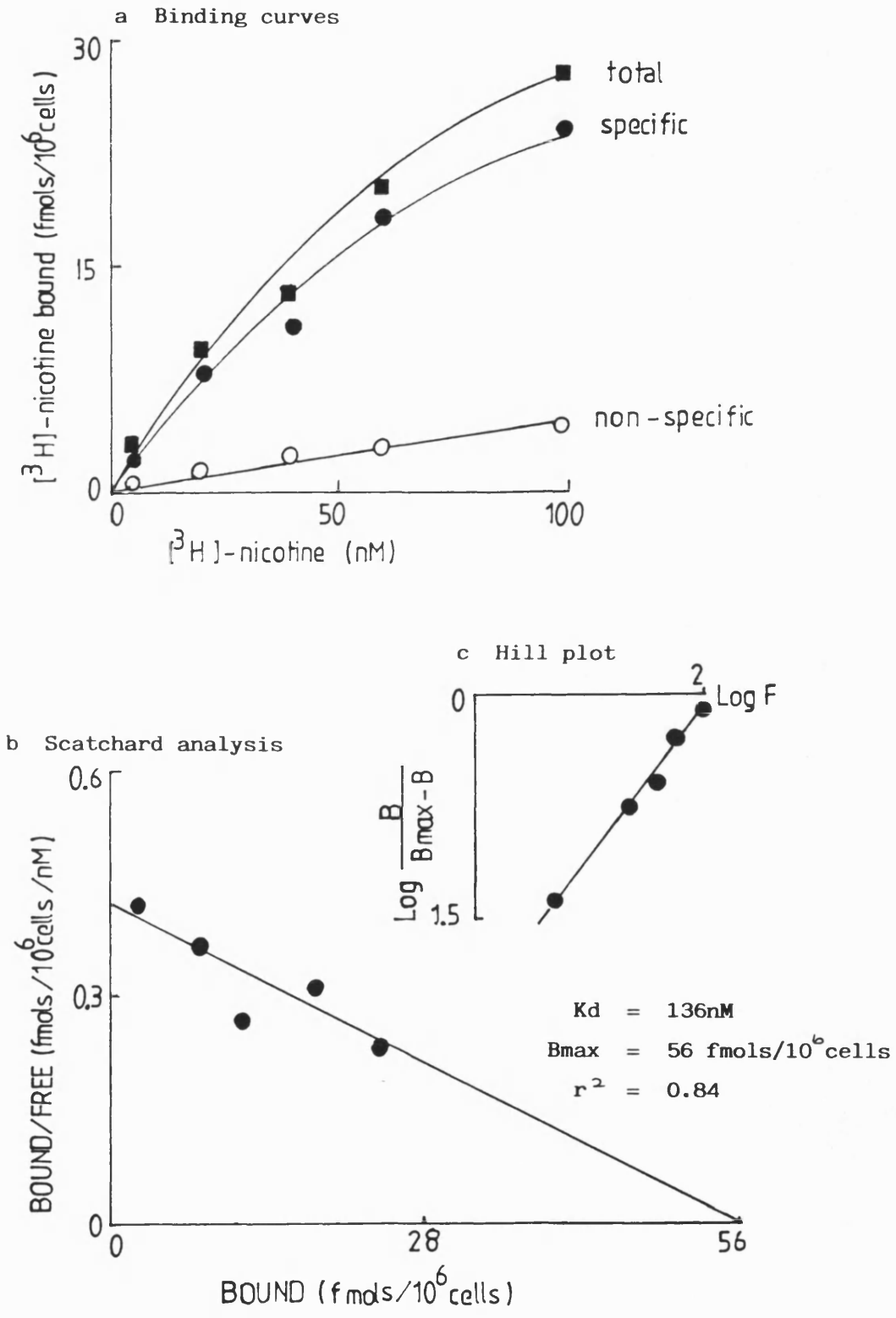


b Human PBL



The results are the mean of triplicate determinations of specific [^{3}H]-(-)-nicotine binding at 10nM.

Figure 43 [^{3}H]-(-)-nicotine binding to mouse thymocytes



Following this success, no further studies using mouse thymocytes were carried out and attention was focused on [^{3}H] (-)- nicotine binding to human PBL.

2.3.2 [^{3}H] (-)- nicotine to human PBL

2.3.2a Linearity of binding with cell number

The binding of [^{3}H] (-)- nicotine (10nM) to normal human PBL was linear over the range 0 - 1 x 10^6 cells/assay (Figure 42b). All subsequent binding assays were carried out using 0.5 - 1.0 x 10^6 cells.

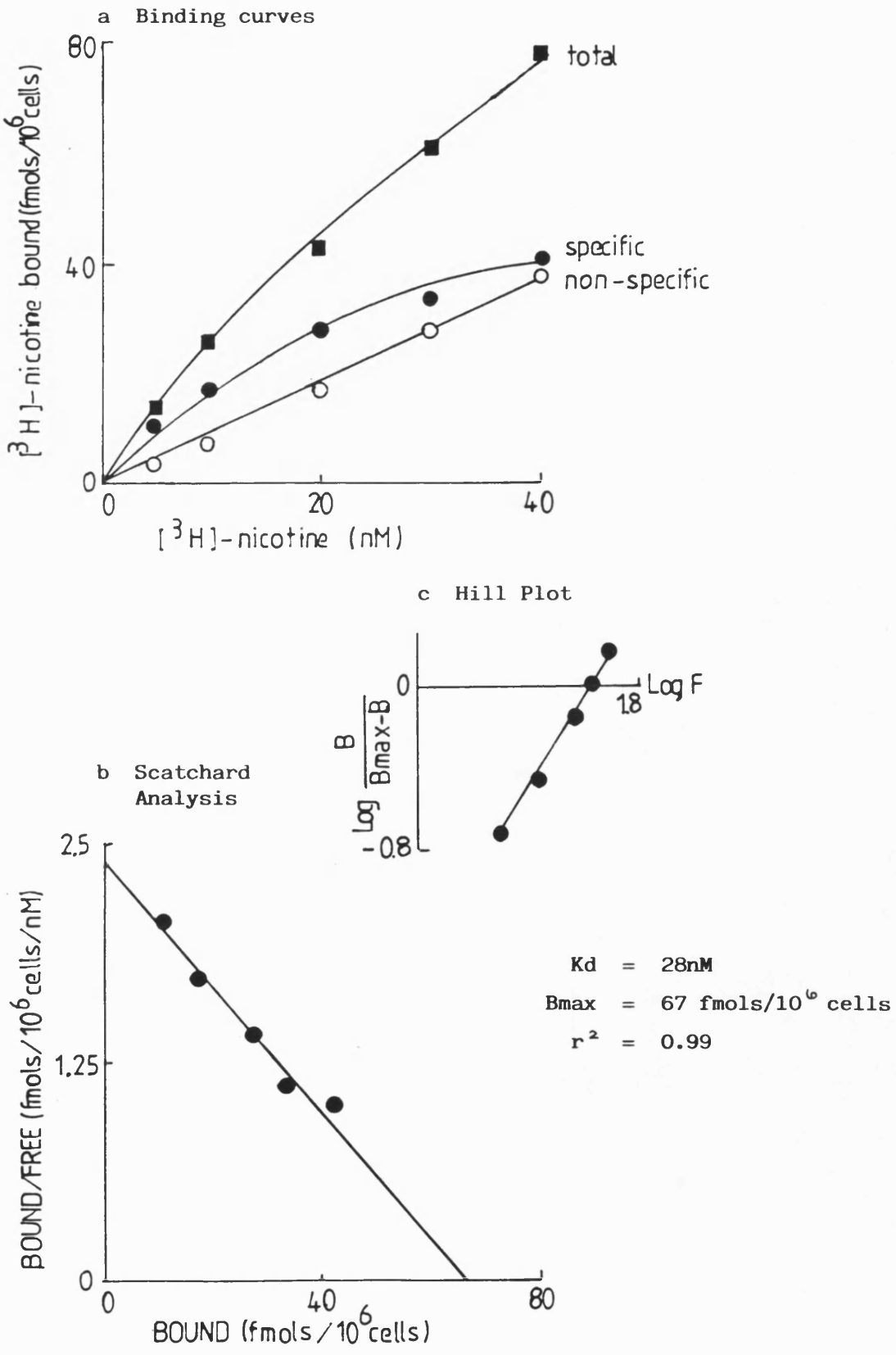
2.3.2b Saturation curves and Scatchard analysis

Saturable specific binding of [^{3}H] (-)- nicotine to isolated human PBL was found (Figure 44). These data are representative of results obtained in 5 experiments using PBL from different donors. Table 35 summarises the data obtained. The mean Kd was 59 $\pm$ 10nM (mean $\pm$ S.E.) and Bmax values of 287 $\pm$ 80 fmols/10^6 cells (mean $\pm$ S.E.).

PBL isolated from leucopheresis samples from two MG patients (see Results, Section D1) were also used in [^{3}H] (-)- nicotine experiments.

The binding of [^{3}H] (-) nicotine to both myasthenic PBL samples was saturable. Figure 45 a gives the saturation curve obtained when PBL from patient 1 was studied. Scatchard analysis of the data (Figure 45 b) gave a Kd of 79nM and Bmax value of 92 fmols/10^6 cells (r^2 = 0.88). Analysis of the data from patient 2 gave a Kd of 65nM and Bmax value of 75 fmols/10^6 cells

Figure 44 [^{3}H]-(-)- nicotine binding to human PBL



Kd = 28nM

Bmax = 67 fmols/10^6 cells

r^2 = 0.99

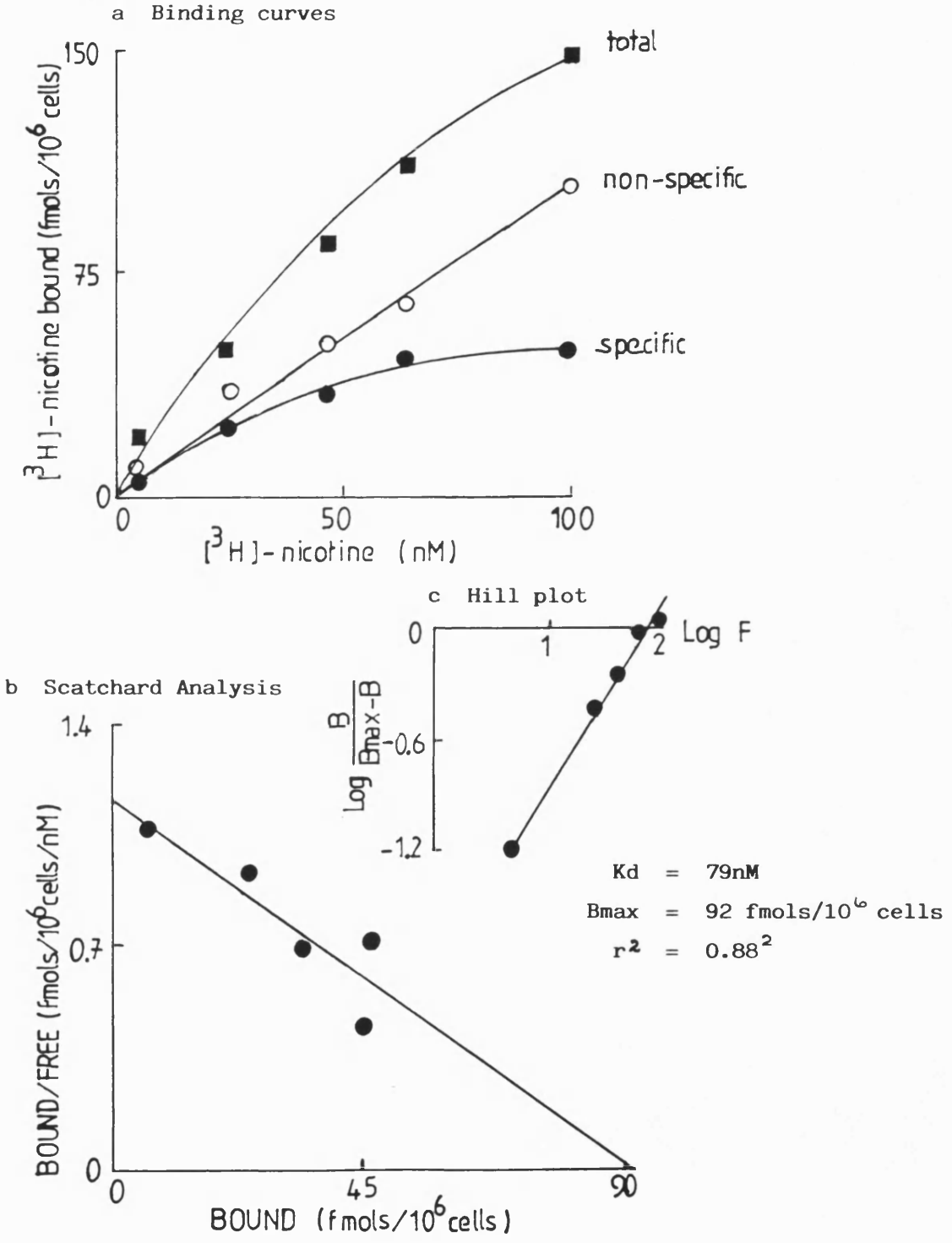
Table 35 Summary of the Binding Data of [^{3}H] (-)-nicotine to intact human PBL

Source of Lymphocytes	Kd nM	Bmax fmols/10^6 cells	correlation coefficient r^2	Hill coefficient N_H
Normal blood	[a]28	67	0.99	0.92
" "	44	157	0.96	0.95
" "	80	225	0.85	0.96
" "	94	555	0.72	0.90
" "	53	435	0.97	0.91
Leucopheresis sample Myasthenic patient 1	79	92	0.88	0.96
Leucopheresis sample Myasthenic patient 2	65	75	0.84	0.94
Mean $\pm$ SE of all data	63 $\pm$ 8.0	229 $\pm$ 67	0.89 $\pm$ 0.03	0.93 $\pm$ 0.01

Data were obtained by using Scatchard analysis.

[a]The saturation curve and Scatchard plot is shown in Figure 44 (page 228).

Figure 45 [^{3}H]-(-)- nicotine binding to Myasthenic PBL (Patient 1)



(r^2 = 0.84). The Hill coefficients were 0.96 and 0.94 respectively (Figure 45 c)

The binding of [^{3}H] (-)- nicotine to normal human PBL was further examined by the determination of the association and dissociation constants.

2.3.2c Determination of the association rate constant for [^{3}H] (-)- nicotine binding to human PBL

The association rate (K + 1) was determined at 23°C, by incubating the assay mixture, human PBL and [^{3}H] (-)- nicotine for increasing times before filtration (see Methods, Section D2.4). The half-time (t 1/2) for association was approximately 1.25min and the binding reached equilibrium within approximately 30min (Figure 46a).

The data were subjected to pseudo first-order treatment which takes into consideration the contribution of ligand-receptor dissociation to the eventual reaching of a steady state of equilibrium (Beq) (see Bennett, 1978).

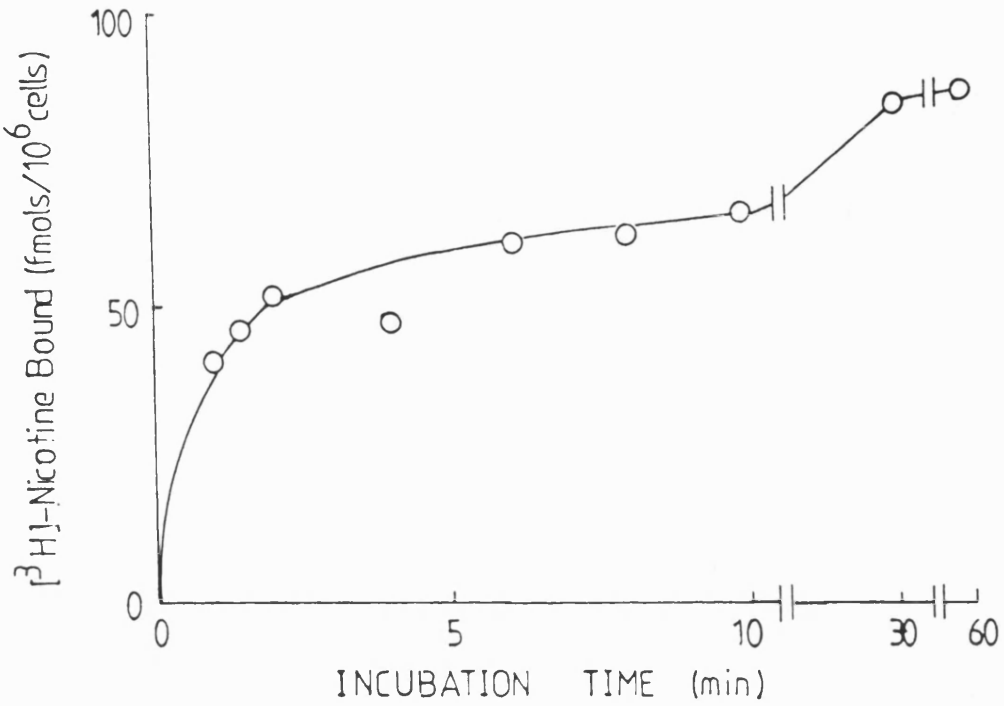
The bound concentration of [^{3}H] (-)- nicotine (Bt) at any prior time is related to the equilibrium concentration (Beq) by the following equation :

$$\text{Ln}\left[\frac{\text{Beq}}{\text{Beq} - \text{Bt}}\right] = (K + 1 + K - 1)\ t$$

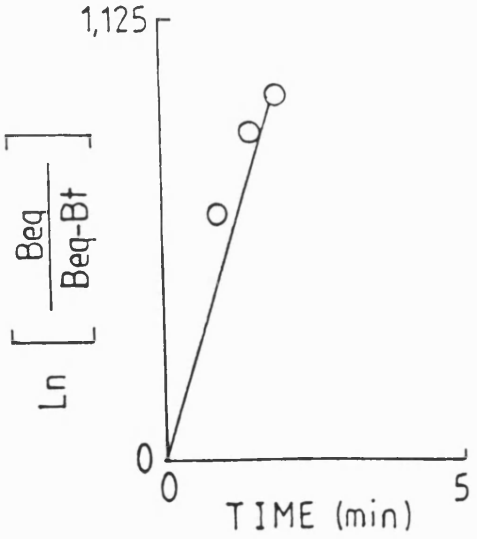
$$= \text{Kobt}$$

Hence, the slope of the plot Ln [Beq/Beq-Bt] versus time gives the experimentally observed apparent rate constant (Kobs) (see Figure 46 b) and was found to be 0.46.

Figure 46a Time course of association of [^{3}H](-)-nicotine with human PBL



46 b Pseudo first-order treatment of data



Kobs = 0·466
r^2 = 0·977

Kobs is represented by the initial slope, determined graphically by least squares linear regression.

Kobs is related to the association (K + 1) and dissociation (K - 1) rate constants and free ligand concentration [L] as follows:

$$K + 1 = \frac{Kobs - K -1}{[L]} \qquad \text{EQUATION 1}$$

Hence, estimation of K + 1 requires knowledge of the dissociation rate constant (K -1). This was determined as described in the next section.

2.3.2d Determination of the dissocation rate constant or $[^3H]$ (-)- nicotine binding to human PBL

The rate of dissociation was determined by incubating human PBL with $[^3H]$ (-)- nicotine at 23°C for 60min (ie: to equilibrium) followed by the addition of excess unlabelled (-)- nicotine as described in Methods, Section D2.4. The amount of $[^3H]$ (-)- nicotine bound was determined at various times thereafter (Figure 47 a).

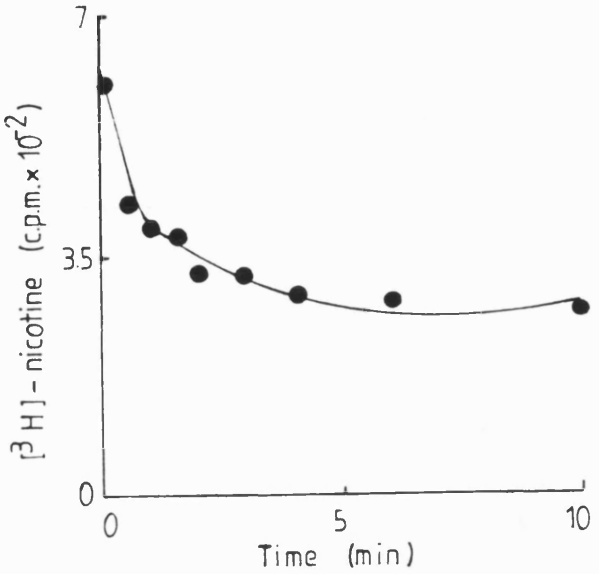
If $B = Bo$ at $t = 0$ then

$$Ln \frac{B}{Bo} = -K -1.t$$

Hence a plot of Ln [B/Bo] vs time has a slope of - K -1 (see Figure 47 b) estimated by linear regression analysis.

The t 1/2 for dissociation was approximately 2 min and the dissociation curve reached a plateau within 6 min. The dissociation rate constant (K-1) was determined graphically to be $0.35min^{-1}$ at 23°C and the association constant (K + 1) as calculated from equation (1) was 5.8×10^{-3} nM^{-1} min^{-1} .

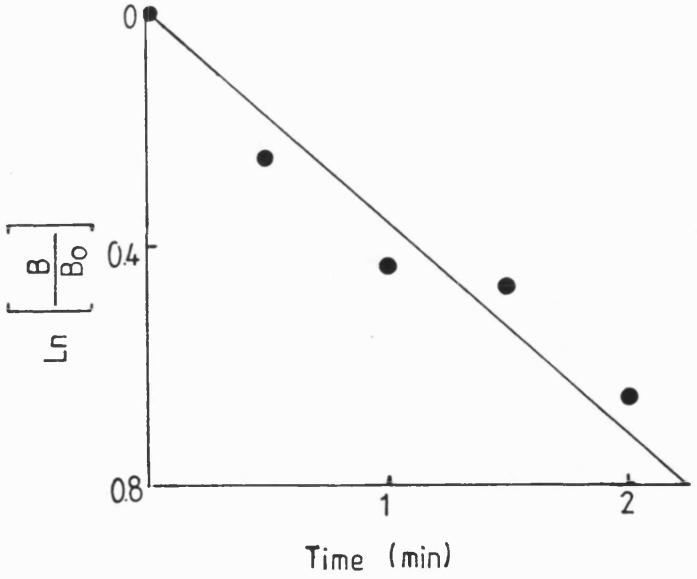
Figure 47a The rate of dissociation of [^{3}H](-)-nicotine to human PBL



The dissociation of [^{3}H](-)-nicotine was determined as described in Methods, Section D 2.4

Each point is the mean of 4 determinations.

47 b First-order kinetic plot of [^{3}H](-)-nicotine specific binding



The dissociation constant K-1 is represented by the negative slope, determined by least-squares linear regression.

The Kd determined by the ratio K-1/K+1 was 60nM, in agreement with the mean Kd determined by equilibrium studies from five PBL samples examined (see Results, D2.3.2).

2.3.3 [^{3}H] (-)- nicotine binding to human granulocytes

The binding of [^{3}H] (-)- nicotine to another population of human leucocytes, namely granulocytes was also investigated.

The binding of [^{3}H] (-)- nicotine to two preparations of granulocytes was saturable. Figure 48 a shows a representative binding curve. Scatchard analysis (Figure 48b) of the data from the two binding curves gave a mean Kd of 40nM and Bmax values of 750 fmoles/10^6 cells, indicating approximately 450,000 binding sites per cell. Hill coefficients for the two binding curves were 1.04 and 0.98(Figure 48c).

Granulocytes were also found to be a minor contaminant (representing approximately 2%) of PBL population (see Table 32) Hence a 2% contamination by granulocytes would contribute approximately 15 fmols/10^6 cells of [^{3}H] (-)- nicotine binding sites to the estimation of [^{3}H] (-)- nicotine binding sites on human PBL.

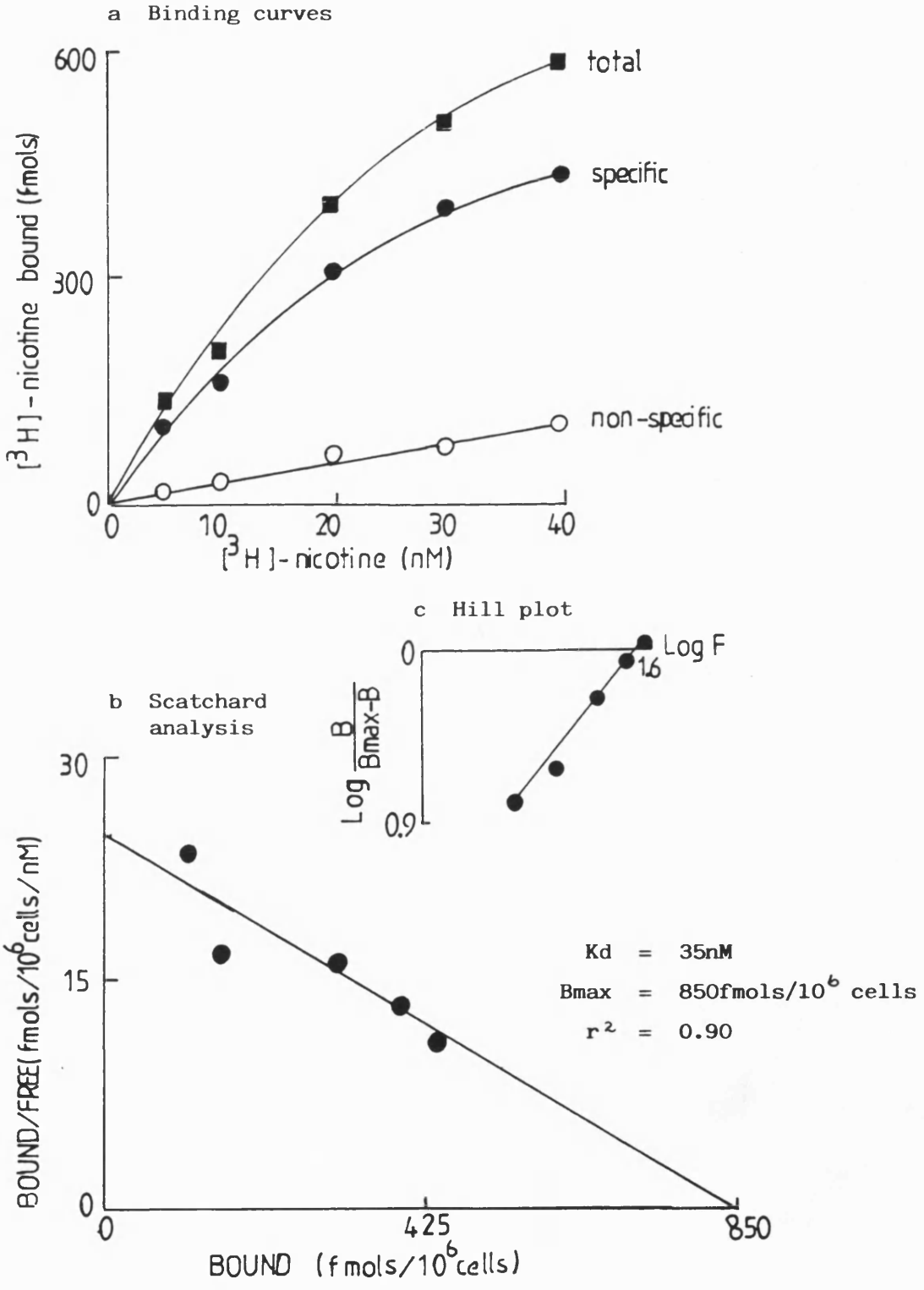
2.3.4 [^{3}H] (-)- nicotine binding to human erythrocytes

No specific binding of [^{3}H] (-)- nicotine to human erythrocytes was observed over the concentration range 0-60nM.

2.4 Competition Studies

The binding of [^{3}H] (-)- nicotine binding to human leucocytes was further characterised with respect to stereoselectivity and

Figure 48 [^{3}H]-(-)-nicotine binding to granulocytes



pharmacological profile.

The ability of anti -(nAChR) monoclonal and polyclonal antibodies (described in Results, Section D2.4.3) to inhibit [^{3}H] (-)- nicotine binding to human PBL was also investigated.

2.4.1 Stereoselectivity of [^{3}H] (-)- nicotine binding

Two different tartrate salts (Nicotine hydrogen (+) tartrate and Nicotine (-) ditartrate) of (-) and (+) nicotine were compared for inhibition of [^{3}H] (-)- nicotine binding to human PBL (Figure 49b).

IC50 values (concentration of drug that inhibits specific binding by 50%) were derived from linear transformations of dose response curves (see insert Figure 49c) and were 5.0×10^{-5} M for (+)-nicotine and 1×10^{-4} M for (-)-nicotine. Hence the inhibition by (+) and (-)-nicotine showed little stereoselectivity.

Assuming the Kd to be approximately 60nM (see Results, Section D2.3.2) the Ki values can be derived from IC50 values by the following equation (Cheng and Prusoff, 1973):

$$Ki = \frac{IC50}{1 + \frac{[L]}{Kd}}$$

where [L] is the concentration of radioligand used in the displacement study

Hence the Ki values for (-) and (+) are 7.5×10^{-5} M and 3×10^{-5} M respectively.

In a comparative study the inhibition of [^{3}H] (-)-nicotine binding to rat brain P2 membranes by nicotine (-) ditartrate was

Figure 49a Inhibition of [^{3}H] (-)-nicotine binding to rat brain P2 membranes

Diluted rat brain P2 membrane was preincubated for 5 min with concentrations of nicotine indicated on the abscissa before addition of 40nM [^{3}H] (-)-nicotine. Assays were carried out as described in Methods, Section P2.3 Non-specific binding, determined in the presence of 10^{-3} M (-) nicotine was subtracted from all values, binding in the presence of test concentrations of drug was calculated as a percentage of the total specific binding to the preparation. Data points are the means of triplicate determination of one experiment.

—□— (-)-nicotine ditartrate.

Figure 49b Stereospecificity of inhibition of [^{3}H](-)-nicotine to human PBL by nicotine

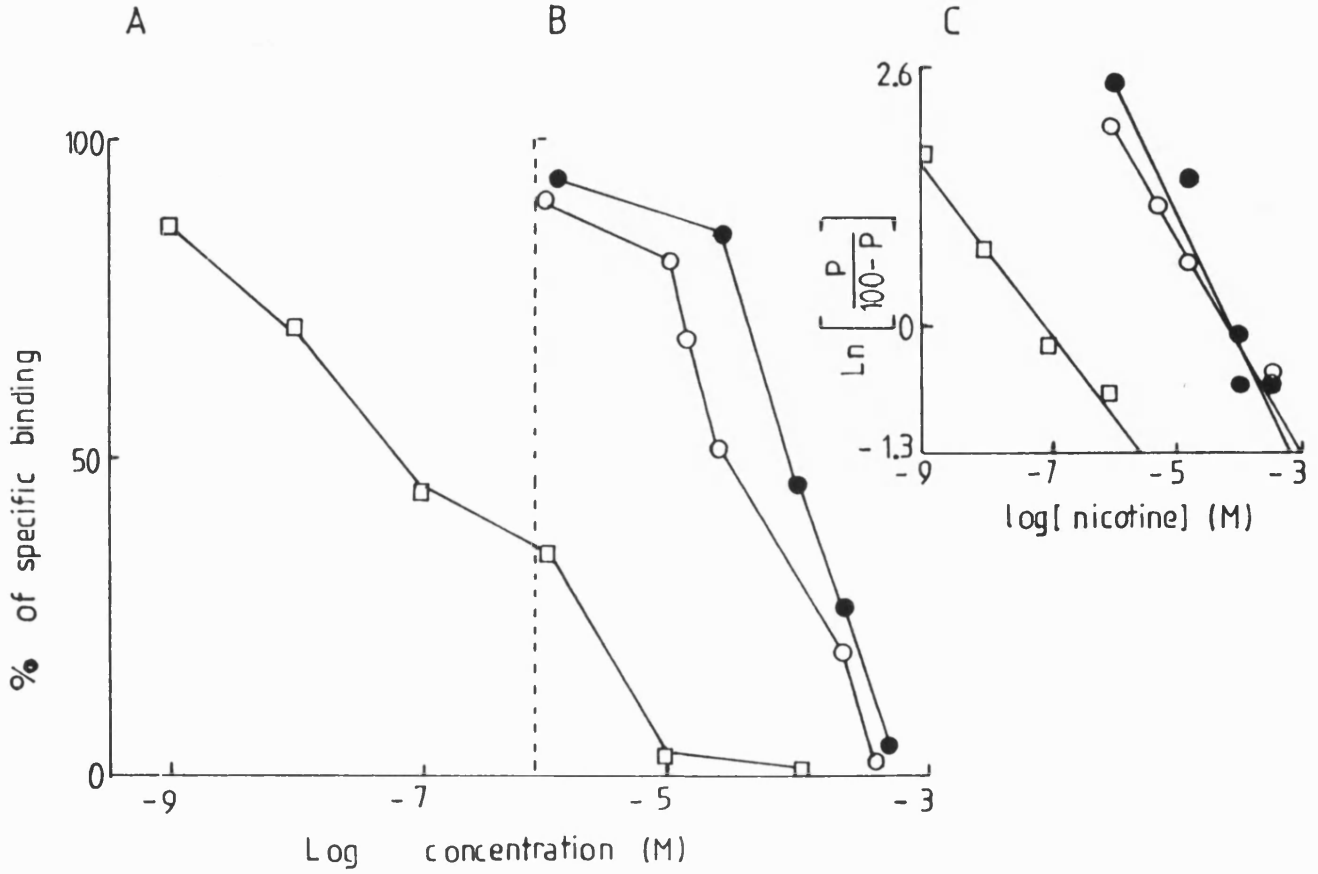
The conditions used were exactly the same as above except that human PBL were used and 30nM [^{3}H](-)-nicotine was used.

Data points are the means of triplicate determinations and the curves are representative of three experiments on different PBL preparations.

—●— (-)-nicotine ditartrate;
—○— nicotine hydrogen (+) tartrate.

Figure 49c Linear transformation of the dose response curves shown in Figure a and b where P represents the percentage bound at each concentration of nicotine.

FIGURES 49


A
B
C
100
50
0
% of specific binding
-9
-7
-5
-3
Log concentration (M)
2.6
0
-1.3
Ln [P / 100-P]
log[nicotine] (M)

examined (Figure49a). A much lower range of nicotine concentrations were used than for the displacement studies above. Linear transformation of the displacement curve binding data gave an IC50 value of 1×10^{-7}M. [^{3}H] (-)- nicotine binding to rat brain P2 membranes is well characterised in the laboratory and assuming a Kd value of 50nM (see Wonnacott, 1986), the Ki value calculated as above was 37nM.

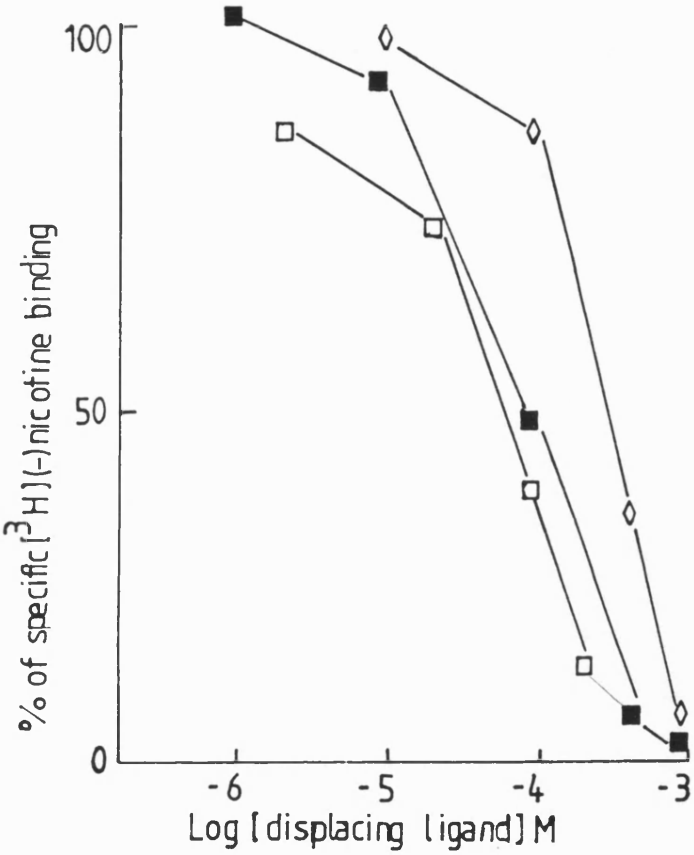
2.4.2 Pharmacological specificity of [^{3}H] (-) nicotine binding to human PBL

The pharmacological specificty of [^{3}H] (-)- nicotine binding to human PBL was studied by examining the inhibition of specific [^{3}H] (-)- nicotine binding by various drugs (Figure50, Table 36 and 37).

Three cholinergic drugs were effective in displacing [^{3}H] (-)- nicotine binding, the nicotinic antagonists d-tubocurarine, dihydro-β-erthyroidine and atropine, a muscarinic antagonist. The IC50 values were estimated by using a linear transformation of the data, as described in Figure 50 , page241 , and are summarised in Table36 .

Other well established nicotinic agonists, acetylcholine carbachol and DMPP and the antagonists, α-BGT, hexamethonium and decamethonium (see Introduction 2.2, page4) failed to inhibit [^{3}H] (-)- nicotine binding to human PBL (Table 37). Initial experiments using acetylcholine used physostigmine (10^{-3}M) as anticholinesterase agent, however this drug alone inhibited [^{3}H] (-)- nicotine binding (10^{-3}M physotigmine

Figure 50 Competition of cholinergic ligands for [^{3}H](-)-nicotine binding.



The conditions used were exactly the same as described for Figure 49 . Data points are the mean of triplicate determinations and are representative of three experiments carried out on different human PBL samples.

-◇- d-tubocurarine, -■- atropine,
-□- dihydro-β-erythroidine.

Stock dihydro-β-erythroidine was prepared in ethanol. Controls using ethanol alone had no effect on [^{3}H](-)-nicotine binding.

producing 50% inhibition). An alternative anticholinesterase agent BW 284C51 which had no effect on [^{3}H] (-)- nicotine binding to human PBL was used in subsequent experiments.

The inhibition profile of [^{3}H](-)- nicotine binding to human granulocytes was similar to that for human PBL.

Table 36 Inhibition of [^{3}H] (-)- nicotine binding to human PBL using cholinergic ligands

Ligand	IC50
nicotine (-) ditartrate	1.0×10^{-4} M
nicotine hydrogen (+) tartrate	5.0×10^{-5} M
d-tubocurarine	2.5×10^{-4} M
atropine	8.0×10^{-5} M
dihydro -β- ethyroidine	5.0×10^{-5} M

Table 37 Cholinergic ligands which showed no inhibition of [^{3}H] (-)- nicotine binding to human PBL

Ligand
Acetylcholine[a]
Decamethonium
DMPP[b]
Carbachol
Hexamethonium
α-BGT[c]

[a] determined in the presence of the acetylcholinesterase BW 284C51 (10μg/ml); [b] DMPP 1-1-dimethyl-4-phenyl piperazinium iodide was used at 10^{-4} M; [c] α-BGT was used at 10^{-4} M. All other ligands were used at 10^{-3} M.

2.4.3 Inhibition studies using anti-(AChR) monoclonal and polyclonal antibodies

The polyclonal and monoclonal antibodies described in Section C were examined for their ability to inhibit [^{3}H] (-)- nicotine binding to rat brain P2 membranes and human PBL (see Methods, Section D2.5). None of the antibodies inhibited [^{3}H] (-)- nicotine binding when compared to the control values using irrelevant antibodies. All the antibodies inhibited [^{3}H] (-)- nicotine binding by approximately 10%.

2.5 Effect of heat on binding of [^{3}H] (-)- nicotine to human PBL

Several other studies were carried out to investigate the anomolies of [^{3}H] (-)- nicotine binding to human PBL. These anomalies are : lack of correlation between IC50 values and observed Kd, lack of stereoselectivity and the lack of significant inhibition of [^{3}H] (-)- nicotine binding by classical nicotinic agents.

2.5.1 Heat inactivation of [^{3}H] (-)- nicotine binding to human PBL

The binding of [^{3}H] (-)- nicotine (20nM) to human PBL was sensitive to heat treatment, with 36% of the specific binding, observed at 22°C, remaining after incubating the cells at 60°C for 5min. The specific binding was totally obliterated after heat treatment for 5 min at 100°C. Table 38 summarises the results.

2.5.2 Effect of incubation temperatures on [^{3}H] (-)- nicotine binding

The binding of [^{3}H] (-)- nicotine to human PBL was compared at

4°C and 22°C. Human PBL from the same donor were either pre-cooled on ice to 4°C or left at room temperature (22°C). Incubation with [^{3}H] (-)- nicotine (40nM) for 30 min was carried out at these temperatures. Table 39 gives the results. There was no difference between the specific binding observed at 4°C and 22°C.

Table 38 Heat inactivation of [^{3}H] (-)- nicotine binding to human PBL

Temp.	Total binding c.p.m.	non-specific c.p.m.	specific c.p.m.
22°C	2221 ± 29	1351 ± 80	870
60°C	1669 ± 20	1358 ± 100	311
100°C	1661 ± 50	1530 ± 80	12

Non-specific binding was carried out in the presence of 10^{-3} M(-) nicotine. 1.2 x 10^6 cells were used for each assay tube. Results are expressed in c.p.m.

Table 39 Comparison of incubation temperature on [^{3}H] (-)- nicotine binding to human PBL

Incubation at 4°C			Incubation at 22°C		
Total binding c.p.m.	non-specific c.p.m.	specific c.p.m.	total binding	non-specific c.p.m.	specific c.p.m.
5426 ± 129	2038 ± 139	3338	5321 ± 141	1851 ± 40	3470

Non-specific binding was carried out in the presence of 10^{-3} M(-) nicotine. 1.8 x 10^6 cells were used per assay tube and determinations are the mean ± S.E. of triplicates. Results are expressed as c.p.m.

2.6 Binding of [^{3}H] (-)-nicotine to subcellular fractions of human PBL

The binding of [^{3}H] (-)- nicotine to lymphocyte membranes and detergent extracts of lymphocytes was determined in the following studies.

2.6.1 [^{3}H] (-)- nicotine binding to detergent extract of lymphocytes

A detergent extract of human PBL was assayed for [^{3}H] (-)- nicotine binding sites (Methods, Section D1.5). Detergent extracts prepared from two PBL samples showed no specific binding of [^{3}H] (-)- nicotine. The presence of detergent at 0.1% (v/v) had no effect on the binding of [^{3}H] (-)- nicotine to rat brain P2 membranes (see Table 40 page 248).

Human PBL or rat brain P2 membranes were incubated with [^{3}H] (-)- nicotine in assay buffer (Methods, Section D2.3) or assay buffer containing additionally 0.1% (v/v) Triton X-100 or 0.01% (v/v) Triton X-100. The presence of detergent had little effect on the specific binding of [^{3}H] (-)- nicotine to rat brain P2 membranes, but reduced the binding of [^{3}H] (-)- nicotine to human PBL to approximately 22% of the binding found in buffer. Table 40 summarises the results. In a parallel experiment, human PBL were incubated with buffer and 0.1% (v/v) Triton X-100 alone and samples (30 µL) were stained with Trypan Blue (Methods, Section D1.1) and examined under the light microscope. The PBL remained intact in buffer but only approximately 15% of the cells were intact from the PBL/detergent sample.

2.6.2 [^{3}H] (-)- nicotine binding to lysed lymphocytes

One PBL preparation in buffer was repeatedly frozen in liquid N2 and thawed at room temperature in order to determine [^{3}H] (-)- nicotine binding to lysed lymphocytes. However, this procedure was unsuccessful because the lysed cells produced a solid mass which was difficult to disrupt. Alternative methods of determining [^{3}H] (-)- nicotine binding to lymphocyte membranes were thus investigated.

Lymphocyte membranes were prepared from human PBL using hypo-osmotic shock, followed by homogenisation and centrifugation (see Methods, Section D1.5). Lymphocyte membranes and intact lymphocytes from the same donor were used in [^{3}H] (-)- nicotine binding experiments. Lymphocyte membranes, equivalent to 4 x 10^6 cells were used for each determination whilst assays using intact lymphocytes employed 1 x 10^6 cells.

No specific binding of [^{3}H] (-)- nicotine (40nM) was observed to three preparations of lymphocyte membranes although specific binding of [^{3}H] (-)- nicotine was found as described above (Results, Section D2.3.2) in parallel experiments using intact lymphocytes.

In an alternative approach intact lymphocytes were incubated with [^{3}H] (-)- nicotine and then lysed on the filter using a water wash step. Parallel experiments used buffer washes only (see Methods, Section D2.3).

An initial experiment, using rat brain P2 membranes in the radioligand binding assay was carried out to investigate the effects of a water wash on [^{3}H] (-)- nicotine binding. Table 40 summarises the results. A water wash did not alter the binding of [^{3}H] (-)- nicotine to rat brain P2 membranes. However, the specific binding of [^{3}H] (-)- nicotine to human PBL was apparently abolished. When the binding of [^{3}H] (-)- nicotine to human PBL was systematically studied, over a range of concentrations of radioligand, specific binding to human PBL was observed but was abolished by washing with water.

The study of [^{3}H] -QNB binding to human PBL (Results, Section D2.2) had also used intact lymphocytes. To examine whether cell lysis affected the binding of [^{3}H]-QNB (30nM), parallel experiments were set up, as above, using buffer wash and water wash steps (see Table 40). Specific binding to PBL was still detectable after lysis but was reduced to 21% of that found using intact lymphocytes.

Table 40 Effect of Lysis by Hyposmotic shock or treatment with detergent on [^{3}H] (-)-nicotine binding to lymphocyte and Rat brain P2 membranes

Membrane Source	Radio ligand	Treatment	Specific binding (c.p.m.)
Rat Brain P2	[^{3}H] (-)-nicotine	Buffer Wash	892
		Water Wash	803
		Buffer Incubation	1387
		Buffer + 0.1% (v/v) Triton X-100	1246
		Buffer + 0.01% (v/v) "	1260
[a] Human PBL	[^{3}H] (-)-nicotine	Buffer Wash	638
		Water Wash	12
		Buffer Incubation	775
		Buffer + 0.1% (v/v) Triton X-100	176
		Buffer + 0.01% (v/v) "	182
[b] Human PBL	[^{3}H] -QNB	Buffer Wash	6001
		Water Wash	1279

[^{3}H] (-)-nicotine was used at 10nM and [^{3}H] QNB at 30nM.
Rat brain P2 membranes were used at 1.2mg/ml
[a] Human PBL used at 2.0 x 10^6 cells/assay
[b] " " " " 1.2 x 10^6 cells/assay
The filtration assays employed water as a washing agent (lysed lymphocytes) and buffer as a washing agent (Intact lymphocytes). (see Methods, Section D2.3).

DISCUSSION

Despite the detection, by using iodinated α-BGT, of AChR - bearing myoid and epithelial cells in the thymuses from human and animal sources (see Introduction, Section 4) there is little information concerning binding of α-BGT to thymic cells. In the present study, saturable specific binding of [^{125}I]-α-BGT was observed in only one of several preparations of CFLP thymocytes. Analysis of the binding suggested a single binding site with an affinity (Kd = 6.3nM, Results, Section D2.1.2b) similar to that of nAChRs from skeletal muscle and electric organ, suggesting a similar receptor type. In contrast, a previous study, using Triton X-100 extracts of rabbit thymus, found two [^{125}I]-α-BGT binding components with respective Kd's, 0.11nM and 2nM (Ueno et al., 1980). Unfortunately it is not possible to compare the receptor content of the thymuses concerned, because of the different approaches. However, the contents are small; in this work the mouse thymus contained approximately 10^{11} [^{125}I]-α-BGT binding sites, equivalent to approximately 400fmols AChR whereas Ueno et al, (1980) reported 0.6pmolα-toxin binding sites/mg protein using thymic extracts.

One disadvantage of using whole thymic cell populations or thymic extracts is that it is impossible to determine which cell type or types are binding [^{125}I]-α-BGT. The thymus contains a heterogeneous population of cells; T cells at different stages of maturation, B cells, follicular dendritic and interdigitating

cells, in addition to myoid and epithelial cells. There are reports of immunoreactivity of anti-AChR antibodies with mouse thymocytes (Fuchs et al., 1980; Horvat et al., 1983; Riviera et al., 1987). However, not all thymocytes appear to bind anti-(AChR) antibody, some 38-80% of the population being positive. Similar results have been reported for human thymocytes (Horvat et al., 1983; Pizzighella et al., 1983b; Riviera et al., 1987). The reasons for the inconsistency of [^{125}I]-α-BGT binding to CFLP mouse thymocytes used in this study are unclear, as the cell suspensions and assays were apparently carried out under identical conditions. Fuchs et al., (1980) were unable to demonstrate any significant toxin binding activity on thymocytes or in thymic extracts, although immuno-reactivity with anti-AChR antibodies was found.

Antibody-reactive AChR thymic components have been found both in mice genetically susceptible to EAMG and those which are not (see Fuchs et al., 1980). Similarly, AChR has been found to be present in both control and MG thymus (Pizzighella et al., 1983b; Matsumoto et al., 1986; Kirchner et al., 1987; Melms et al., 1988). This suggests that the presence of AChR in the thymus is not a pathological feature of EAMG or MG.

Although some functional nAChRs do not bind α-BGT (see Introduction, Section 3.6), α-BGT has been shown to alter the functional response of nAChR on lymphocyte and monocyte populations isolated from normal blood. Thus, α-BGT, at a concentration of 10nM, was shown to abrogate the enhancing

effects of ACh on the production of complement factor C2 by monocytes (Whaley et al., 1981). In the present study, PBL fractions were not depleted of monocytes, which represented 12% of the total leucocyte population used in binding assays (Results, Table 32). In other studies, 10nM α-BGT was able to inhibit lymphocyte-mediated cytotoxicity induced by 0.1nM carbamylcholine (Strom et al., 1974), while Richman and co-workers (1979; 1981) found that α-BGT, albeit at a much higher concentration (10μM), was able to reverse the suppressive effect of nanomolar concentrations of carbamylcholine on PBL proliferation. Together, these studies indicate that α-BGT can interact with the nAChR on human lymphocytes and monocytes and it appears paradoxical that specific [^{125}I]-α-BGT binding sites were not found on normal lymphocytes in this study. However, the number of sites may be very small so as to be undetectable in binding studies. Morrell (1979, 1981) was similarly unable to detect α-BGT-binding, although such sites were apparently present on lymphocytes of untreated MG patients and patients who do not respond to steroid therapy. The MG-specific receptor might represent a structurally different type that binds α-BGT. These findings imply a novel role of steroid in controlling the expression of the protein. It would be interesting to use the lymphocytes from these two groups in the functional assays described by Richman and co-workers (1979; 1981) and Mizuno et al., (1982a) to see if they behave differently.

The situation is complicated by the finding that a functional nAChR may reside on a particular T cell subset (Richman et al., 1981; Mizuno et al., 1982b; Menard and Rola-Pleszczynski, 1983) and that this may be of the suppressor type (Richman et al., 1981; Mizuno et al., 1982b; Menard and Rola-Pleszczynski, 1983). This subset clearly would represent a small proportion of the total lymphocyte population and detection of α-BGT binding sites on such a population would be difficult. Hence fractionation of lymphocyte populations prior to their use in binding assays may be more enlightening.

The evidence for the presence of AChR in the thymus, demonstrated either by iodinated α-BGT or by immunoreactivity with anti-AChR antibodies, is now overwhelming. However, the exact situation is not known and needs to be clarified. It is possible that the α-BGT binding components are neither functional nor typical AChRs; such components are known to exist in the brain (see Introduction, Section 3.6). Furthermore, a variety of cell types exhibit both α-BGT binding and anti-AChR antibody cross-reactivity and it is not clear whether these properties belong to the same protein entity. Double labelling techniques, using, for example, rhodamine labelled α-BGT and fluorescein-conjugated second antibody to detect anti-AChR antibodies along with well characterised fractionated thymic cell populations may help resolve this question.

The function, if any, of thymic AChR is not known. Pizzighella and co-workers (1982) have suggested that the receptor is

involved with cell proliferation, as an anti-AChR binding site directed Mab was able to reverse the inhibition produced by the effects of succinylcholine on the spontaneous uptake of [^{3}H]-thymidine by mouse thymocytes. The data also suggested a link between the receptor and cAMP production. This second messenger is normally associated with hormone receptor systems and has not previously been linked to AChRs. Of interest here, is the finding that thymopoietin, a polypeptide hormone of the thymus, can bind to and compete with, the α-BGT binding site of AChR prepared from T.californica (Venkatasubramanian et al., 1986). Whether thymopoietin is the native ligand of thymic AChR is not known. However, this peptide hormone is able to induce the differentiation of prothrombocytes to thymocytes and to regulate the functions of T lymphocyte (Venkatasubramanian et al., 1986), systems which most likely function through cell surface receptors.

The presence of mAChRs on lymphocytes has been described by many authors, using both binding and functional assays (see Table 41, page 255, Richman and Arnason, 1979, 1981; Strom et al., 1981). In the present work mAChRs were detected on murine erythrocytes and thymocytes and this appears to be the only report of [^{3}H]-QNB binding to these murine cell types. It is, therefore, difficult to make a direct comparison of the results obtained. However, some observations can be made with respect to the findings of other workers. A single high affinity binding site for [^{3}H]-QNB on thymocytes was observed in this work (Results, Section D2.2.1) and this is similar to the

findings of Gordon et al., (1978) using mouse spleen cells (Table 41 , page 255). It would appear, however, that the muscarinic receptors are present at a one thousand fold lower density on spleen cells than on thymocytes. The majority of other workers have also shown that T cells possess more mAChRs than B cells (Shapiro and Strom, 1980; Strom et al., 1981) and that stimulation by the mitogen PHA, specific for T cells, can cause an increase in [^{3}H]-QNB binding (Szélenyi et al., 1987). However, Atweh et al., (1984) found higher binding to B cell preparations than to T cells. Moreover, they detected two [^{3}H]-QNB binding sites on mouse spleenocytes of much lower affinity than that found in the present study or in that of Gordon et al., (1978). One of the sites reported is of a similar Kd to that observed for [^{3}H]-QNB binding to mouse erythrocytes found in this work and although erythrocyte contamination of the spleen cell preparation was reported to be low, a binding site on the red cells may have been detected. The low affinity binding sites observed by Atweh et al., (1984) also showed mixed muscarinic and nicotinic pharmacology, a property shared with the high affinity site reported to be present on human erythrocytes by Aronstram and co-workers (1977). The significance of muscarinic cholinergic receptors on erythrocytes is unclear. In the present study, [^{3}H]-QNB binding sites were detected on mouse erythrocytes but not on human erythrocytes. This latter finding is in contrast to the high affinity site (Kd 1nM) found by Aronstram et al., (1977) on human erythrocyte ghosts. However, it is likely that such a high affinity site

Table 41 Binding of [^{3}H]-QNB to Leucocyte Populations

Cell Type	Kd (nM)	Bmax (sites/cell)	Reference
Murine thymocytes	39	2×10^5	This Thesis
Murine lymphocytes	1	2×10^2	Gordon et al., 1978
	480	N.D.	Atweh et al., 1984
	16000		
Murine erythrocytes	250	2×10^3	This Thesis
Human lymphocytes	69		
	233		This Thesis
Human lymphocytes	67	5×10^4	Zalcman et al., 1981
Human lymphocytes	26	N.D.	Adem et al., 1986 b
lymphocyte membranes	15	N.D.	
Human granulocytes	16	8×10^4	
Sonicated human granulocytes	8	N.D.	Lopker et al., 1980
Human monocytes	20	3×10^4	
[a] Human T cells	1-5	5×10^3	Strom et al., 1981
[b] Human erythrocytes	No Binding		This Thesis
Human erythrocyte membranes	1.3	N.D.	Aronstam et al., 1977
Mouse brain homogenate	0.037	N.D.	Marks and Collins, 1982

All studies were carried out using whole cells unless otherwise stated.

N.D. not determined in publication.

a using T cells activated by Concanavalin A

b no binding observed over the range 0-200nM [^{3}H]-QNB

would not have been detected in this present study because of the concentration range of [^{3}H]-QNB employed. Similarly, a low affinity site found in this work would not have been detected in Aronstram's study.

Investigation of [^{3}H]-QNB binding sites on human PBLs in the present study gave a curvilinear Scatchard plot suggesting either the presence of multiple binding sites or co-operativity. The data, however, were consistent with a two site model by computer analysis and although, in inhibition studies, atropine failed to distinguish between the two sites, Hill plot analysis suggested a lack of co-operativity (Results, Section D2.2.3). Further studies examining association and dissociation rates would help to provide further support for the presence of two populations of receptors. Positive co-operativity has previously been demonstrated in QNB binding studies to human phagocytes (Lopker et al., 1980).

The high affinity [^{3}H]-QNB binding site, Kd = 69nM, observed on human lymphocytes in this work corresponded closely to that observed by Zalcman et al., (1981) although the low affinity site was not observed by these workers. Other workers have similarly found high affinity [^{3}H]-QNB binding sites, not only on lymphocytes but also on human granulocytes and monocytes (Table 41). The presence of two mAChRs which are functionally active at different carbamylcholine concentrations has been described by Richman and Arnason (1979) in <u>in vitro</u> lymphocyte proliferation studies. The receptors appear to be active at

0.1nM and 1 μM carbamylcholine concentrations. Although these concentrations do not correspond to the binding affinities found here, this may be due to the approaches used and the differences in the affinity of the receptor for various cholinergic ligands. Electrophysiological experiments, using murine T lymphocytes have also shown that a wide range of concentrations of ACh and carbamylcholine can produce changes in membrane potential, effects abrogated only by the muscarinic antagonist atropine and not by nicotinic agents. Maximum responses were observed using 10nM ACh and 1nM carbamylcholine although higher concentrations of ACh and carbamylcholine were also effective.

Muscarinic cholinergic stimulation of lymphocytes appears to result in an increase in cGMP levels (Szélenyi et al., 1987) and this increase has ben shown to be directly involved in other muscarinic lymphocyte responses; augmentation of mitogen-induced DNA synthesis and cytotoxicity. Changes in Ca^{2+} concentration are also implicated in these responses and indeed carbamylcholine binding to mAChRs on lymphocytes appears to open voltage insensitive calcium channels, accounting for the changes in membrane potential observed by Shapiro and Strom (1980). It would therefore appear that the mAChR on lymphocytes forms a part of second messenger systems which may control immune responses.

Table 42 IC50 values (M) of [^{3}H]-QNB displacement by atropine in lymphocyte studies

Cell Type	IC50	Reference
Human lymphocytes	2.5×10^{-5}	This Thesis
Human lymphocytes	3.4×10^{-6}	Zalcman et al., 1981
Human lymphocyte	3.0×10^{-5}	Adem et al., 1986b
Human lymphocyte membranes	3.0×10^{-7}	Adem et al., 1986b
Human granulocytes	1.0×10^{-5}	Lopker et al., 1979
Murine lymphocytes	1.5×10^{-6}	Atweh et al., 1984
Mouse brain homogenate	6.0×10^{-9}	Marks & Collins, 1982

The IC50 value obtained in this study for the displacement of [^{3}H]-QNB binding by atropine compared well with those obtained by the majority of other workers (see Table 42), although strict comparison is difficult because of the different assay protocols used. However, it is evident that the muscarinic binding sites on lymphocytes have a much lower affinity for atropine and [^{3}H]-QNB than muscarinic binding sites found in brain (Marks & Collins, 1982, Table 42). mAChRs, in general, are functionally diverse and it is now emerging that there are distinct subtypes of mAChRs. The collective data (Table 42) suggest also multiple types of mAChRs with different affinities for [^{3}H]-QNB, and the possibility exists that the binding sites are not homeogenously distributed and that they may be present on different lymphocyte populations.

The majority of workers have used intact lymphocytes in [^{3}H]-QNB binding studies (Table 41). Intact cells were used in this work as the preparation was simple and quick. However, later studies using hypertonically lysed lymphocytes gave four times less [^{3}H]-QNB binding than the parallel study using whole cells (Results Section D2.6). Two other groups have also reported similar results; Adem et al., (1986b) reported a seven fold reduction, whilst Atweh et al., (1984) showed a five fold reduction of QNB binding to membrane fractions compared with whole cells. In the present work, the binding of [^{3}H]-QNB to whole lymphocytes was found to be saturable and displaceable. Nevertheless the findings overall would suggest that an uptake of [^{3}H]-QNB by the intact cells could have occurred, in addition to ligand binding to the membrane. Gossuin et al., (1984) has described trapping of ligands within neuroblastoma cells.

The results found in this present study obviously indicate a change in Bmax values following cell lysis; whether a concomittant change in kd also occurred was not investigated. Two groups have demonstrated slight reduced but not significantly different kd values using membrane fractions rather than whole cells (Lopker et al., 1980; Adem et al., 1986b see Table41) and also changes in IC50 values (Adem et al., 1986b Table42). As two [^{3}H]-QNB binding sites with high and low affinity were demonstrated on intact human lymphocytes in this work, the effect of lysis on the presence of these sites remains to be investigated.

[^{3}H]-nicotine provided an alternative probe for nAChR on human lymphocytes. The initial characterisation of [^{3}H](-)-nicotine binding to human PBL by Scatchard & Hill plot analysis was encouraging and indicated that neither positive nor negative cooperativity or multiple sites were present (Results, Table 35). Additionally, the binding observed gave kd values compatible with the concentrations quoted in functional studies by Richman et al., (1981; 1nM) and Mizuno et al., (1982a; 5-50nM).

However, it was only by further analysis of this binding by inhibition studies and finally using lymphocyte membranes instead of intact cells (Results, Section D2.6) that the 'binding' of [^{3}H]-nicotine appeared to represent accumulation of trapped ligand within the cells.

The evidence leading to this conclusion, comparison of these findings with those of other workers and the consequences of nicotine accumulation within the cells will be discussed here.

Two other groups have used [^{3}H]-nicotine in the study of binding sites on human lymphocytes (Davies et al., 1982; Adem et al., 1986a) and leucocyte fractions (Davies et al., 1982) and the results are compared in Table 43. The 'binding' observed in the present study gave similar kd values to those found by Davies et al., (1982), but the Bmax value obtained here was ten fold higher than that quoted by Davies et al., (1982) and more closely resembled the Bmax value for human monocytes (see Table 43). The monocytes fraction represented approximately 12% of a typical lymphocyte preparation (Results, Table 32) and

Table 43 Binding of [^{3}H]-nicotine to leucocyte populations

Cell Type	Stereoisomer of [^{3}H]-nicotine used	Kd(nM)	Bmax (sites/cell)	Reference
[a] Human Lymphocytes	-	63	130 x 10^3	This Thesis
Human Lymphocytes	±	30 ± 5	18 ± 4 x 10^3	Davies et al., 1982
Human Neutrophils	±	36 ± 28	87 ± 61 x 10^3	"
Human Monocytes	±	38 ± 10	74 ± 50 x 10^3	"
Human Granulocytes	-	40	450 x 10^3	This Thesis
Human Granulocytes	±	168 ± 2.9	65 ± 17 x 10^3	Hoss et al., 1986
Human Granulocytes	±	7.7 ± 1.6	48 x 10^3	"
Human Lymphocyte Membranes	N.G.	6	-	Adem et al., 1986a
Human Lymphocyte Membranes	-	NO BINDING OBSERVED		This Thesis
Murine Thymocytes	-	136	3.4 x 10^4	This Thesis

[a] mean of all binding data see Results, Table 35

N.G. not quoted in publication

this may account for part of the difference in Bmax values obtained. In the studies by Davies and co-workers (1982), binding of [^{3}H]-nicotine was carried out by using whole cells; binding to leucocyte membrane fractions was not examined. Both the present study and the work of Davies et al., (1982) contrast with the results of Adem et al., (1986) where non-saturable binding was observed over the range 5-60nM [^{3}H]-nicotine using intact lymphocytes whereas using lymphocyte membrane fractions a high affinity site was demonstrated. In the present work, no binding to membrane preparations was detected.

Several other properties of the 'receptor' system studied in this work differed from those described previously by other groups. Firstly, the 'binding' observed here showed little stereoselectivity, with the (+) enantiomer being only slightly more potent than the (-) isomer. These findings are in sharp contrast to those with the nAChR of brain, which is known to be stereoselective for the naturally occurring (-) isomer; (+) nicotine being generally 80 times weaker in competition studies (see Wonnacott, 1986). This leads to a 2 fold difference in kd between racemic mixture and [^{3}H](-)-nicotine. Similar differences in kd values, when using optically pure and racemic radioligand mixtures, have been obtained by Hoss et al., (1986) studying the nicotine receptor on granulocytes. However, in this case, the receptor appears to be selective for the (+) isomer of nicotine (Hoss et al., 1986; see Table 43 , page 261.

The present study also demonstrated a discrepancy of approximately four orders of magnitude between the observed kd for [^{3}H](-)-nicotine binding to intact lymphocytes and the Ki value obtained for inhibition by (-) nicotine.

Parallel experiments, using rat brain P2 membrane, gave good correlation (Results, Section D2.4.1). It has been shown that, for the accurate determination of Ki values, it is important that the concentration of receptor sites should not exceed 10% of the kd for the labelled ligand (Jacobs et al., 1975). These conditions were met in this study, where the observed kd was 60nM and the range of concentration of 'sites' was approximately 0.5-0.9nM. Other workers have shown good correlation between IC50 values and kd values (see Table 42 and 44) using intact neutrophil (Davies et al., 1982) and granulocyte populations (Hoss et al., 1986) and human lymphocytes membranes (Adem et al., 1986a).

The pharmacological profile of the 'binding' site on human lymphocytes obtained by inhibition studies demonstrated non-cholinergic properties, in that classical nicotinic agonists failed to compete for [^{3}H](-)-nicotine binding (Results, Section D2.4.2). Similar non-cholinergic properties of a nicotine binding site have also been described by Davies et al., (1982) on human phagocytic leucocytes and by Hoss et al., (1986) on human granulocytes. Nicotinic antagonists, in general, are known to be weak inhibitors of [^{3}H](-)-nicotine binding to central nAChRs and IC50 values of greater than 5×10^{-5} M have

Table 44 IC50 values for nicotine inhibition

Cells	Nicotine Isomer	Stereoisomer of [^{3}H]-nicotine used	IC50	Reference
Human Lymphocytes	-	-	1×10^{-4}	This Thesis
Human Lymphocytes	+	-	5×10^{-5}	This Thesis
Human Neutrophils	-	+	8.0×10^{-7}	Davies et al., 1982
	+	-	2.8×10^{-8}	
Human Granulocytes	-	+	5.2×10^{-6}	Hoss et al., 1986
	+	+	5×10^{-8}	
Human Lymphocytes Membranes	N.G.	N.G.	2×10^{-8}	Adem et al., 1986a

N.G. Not quoted in publication

been reported for d-tubocararine and the muscarinic antagonist atropine and both these drugs showed weak but similar inhibition of [^{3}H](-)-nicotine 'binding' to human lymphocytes (Results, Table 36). Adem et al., (1986a) have also demonstrated inhibition of [^{3}H]-nicotine binding to human lymphocyte membranes by atropine (IC50 3 x 10^{-4}M) and d-tubocurarine (IC50 2 x 10^{-6}M). However, a full pharmacological profile, including nicotinic agonists was not quoted. A third nicotinic antagonist dihydro-β-erythroidine, known to be a potent competitor for the central nAChR, also gave weak inhibition. However, all the drugs that inhibited showed similar IC50 values, indicating a non-discrimatory receptor site.

It would appear from these studies that a pyrrolidine ring structure was critical for inhibition; this structure being common to nicotine, atropine and dihydro-β-erythroidine. This was further suggested by the lack of inhibition by 2-methylpiperdine but inhibition by pyrrolidine and proline (Results, Section D2.4.2). For structures see Appendix 1. Nicotine, at the pH of the assay system, would exist in a monoprotonated form and the presence of the basic N atom may also be an important consideration, since d-tubocurarine does not contain the pyrrolidine ring structure but is diprotonated.

The observed Kd values obtained for [^{3}H](-)-nicotine 'binding' to human lymphocytes was verified by the study of association (K +) and K - 1) dissociation constants (Results, Section D2.3).

Neither of the groups discussed above have used this method to verify the Kd values observed, so a comparison is not possible. Kinetic parameters have, however, been analysed for brain nAChRs (Marks and Collins, 1982). Association rates are mainly dependent on the rate of diffusion of the radioligand to the receptor site and the kinetics of [^{3}H](-)-nicotine binding to lymphocytes could be described as a single first-order process. The dissociation rate is obviously affected by temperature and it is thus difficult to compare values for K-1 at temperatures different to the one used in this work. However, the dissociation rate observed in the present work ($K - 1 = 0.35$ min^{-1}) was similar to that quoted by Marks and Collins, (1982; $K - 1 = 0.282 \pm 0.048 min^{-1}$) for mouse brain nAChR. Human neutrophils also appear to display apparent first order off rate kinetics with a t1/2 of approximately 20min, (Davies et al., 1982), corresponding to a $k - 1 = 0.034 min^{-1}$) using the equation $k - 1 = 0.693/t1/2$ (see Bennett, 1978), but this was carried out at 4^{o}C.

There are, however, differences in the dissociation curves; in the study of Marks and Collins (1982) less than 10% of the radiolabel was remaining after 10 min whereas in this present study approximately 45% of the ligand remained over the same time period at the same temperature. Similar results were obtained by Maslinski et al., (1980) using [^{3}H]-acetylcholine as a ligand for nAChRs on rat lymphocytes where only 50% of the radioactivity was dissociable.

If the observed 'binding' of [^{3}H](-)-nicotine represents the influx of [^{3}H](-)-nicotine into the lymphocytes, how can one account for the saturation curves, dissociation, association kinetics and inhibition curves? Firstly, if all the tests are carried out in the presence of the same cold competing ligand which has the same properties as the radioligand then specific binding is observed in the same way as binding to membrane receptors except that uptake is measured. Hence the parameters being measured were Km and Vmax for the influx of [^{3}H](-)-nicotine. The association rate represents the time necessary for the influx of [^{3}H](-)-nicotine into the cells, whereas the dissociation rate probably represents the equilibration of cold nicotine outside and labelled nicotine inside; hence full dissociation was not observed, and was only observed to a maximum of approximately 50% (Results, Section D2.3.2d). The initial dissociation rate observed would hence represent the rate of flux of cold and labelled nicotine.

Inhibition curves would be observed because as the concentration of competing cold nicotine was reduced, more radiolabelled nicotine would enter. Similarly, other molecules which have properties in common with nicotine would behave in the same manner.

The pharmacological profile of this 'site' on human lymphocytes was similar to that described by Davies et al., (1982) on human phagocytic leucocytes in that both showed non-cholinergic properties and specificity for the pyrrolidine ring structure.

The same properties were also observed for human granulocytes in the present study, a system directly comparable to that described by Hoss et al., (1986). In the latter study and in the work presented here, the binding of [^{3}H](-)-nicotine to granulocyte membranes was not studied. These studies would help confirm that true binding of radioligand was being observed and not uptake as demonstrated in the present study using intact human lymphocytes.

The final experiments, using lysed lymphocytes, did indeed indicate that the 'binding' of [^{3}H](-)-nicotine to human lymphocytes was uptake of radioligand. The process, however, appeared not to be an active process as it was not affected by low temperature (Results, Table 39). This could have been further verified by the use of sodium azide. Interestingly there was no binding to human erythrocytes which could control for the lymphocytes acting as membrane sacs. However, whether this uptake is unique to lymphocytes is unclear. Heat inactivation studies can provide information on the physical state and environment of ligand binding sites and in this study binding or uptake was found to be highly thermolabile, a finding not surprising as denaturation of the lymphocyte membrane and reduction of cell volume with higher temperatures would reduce uptake.

Nicotine is a hydrophobic molecule and would readily seek the membrane environment. However, it would appear from these results that the ligand penetrates the membrane readily.

Nicotine has previously been shown to affect cell membranes and electron-spin resonance studies have demonstrated that (-)-nicotine can produce perturbation of granulocyte membranes at micromolar concentrations (Gala et al., 1984). Nicotine also causes aggregation of human blood platelets (Werle and Schievelbein, 1965), and here nicotine was thought to enhance the permeability of the platelet membrane by changing its charge. Nicotine has also been shown to be a chemotactic agent for neutrophils and enhances neutrophil responsiveness to chemotactic peptides by a process which is thought to affect membrane fluidity (Totti et al., 1984).

The uptake of nicotine by lymphocytes also has consequences for the smoking habit as nicotine is known to reach plasma concentrations of 30-300nM in cigarette smokers; levels at which, according to results shown here, nicotine uptake would occur. Two groups have studied the suppressive effects of nicotine in lymphocyte responses to the mitogen PHA (Neher, 1974; Menard and Rola-Pleszcznski, 1983). In the latter study, effects were observed as nM concentrations, and could be blocked by the addition of myasthenic serum. In this study no inhibition of [^{3}H](-)-nicotine uptake was observed using polyclonal or monoclonal anti-(AChR) antibodies. The effect of nicotine appears to be primarily on DNA synthesis and not on the process of lymphocyte stimulation or cell survival, (Neher, 1974). Hence, it would appear that smoking can adversely affect the immune response.

SECTION E

CROSS-REACTIVITY OF POLYCLONAL AND MONOCLONAL ANTI-AChR ANTIBODIES WITH HUMAN PBL

METHODS

1. Cross-reactivity studies using Solid Phase Radioimmunoassay and ELISA techniques.

1.1 Preparation of fixed PBL coated plates.

1.2 Solid Phase Radioimmunoassay.

1.2.1 Iodination of Protein A.
1.2.2 Determination of the biological activity of Protein A.
1.2.3 Solid Phase Radioimmunoassay protocol.

1.3 ELISA using fixed cell coated plates.

2. Immunoprecipitation of iodinated PBL membranes by anti-(AChR) antisera and Mabs

2.1 Lactoperoxidase-catalysed iodination of lymphocyte plasma membranes.

2.2 Solubilisation of [^{125}I] lymphocytes.

2.3 Trichloroacetic acid precipitation.

2.4 Immunoprecipitation assay.

1. Cross-reactivity studies using Solid Phase Radioimmunoassay and ELISA techniques

The cross-reactivity of anti-(AChR) antibodies with human PBL was studied using Solid Phase Radioimmunoassay and ELISA techniques. These methods required the preparation of fixed PBL coated plates.

1.1 Preparation of fixed PBL coated plates

The preparation of fixed PBL coated plates provided the common target material for the Solid Phase RIA procedure and ELISA technique and will thus be described first.

PBLs were prepared as described in Methods, Section D1.

The cells were washed twice with PBS containing 2% (w/v) BSA and 0.002% (w/v) sodium azide by centrifugation (200g, 10 min, 23°C) and were finally resuspended in the same buffer to 2×10^6 cells/ml. Samples (100 µl) of this cell suspension were added to each well of microtitre plates which had previously been incubated (1h, 23°C) with Concanavalin A (Con A) solution (1mg/ml, 100 µl/well) and washed twice with PBS. Following incubation (2h, 37°C) of the cell suspension with the Con A coated plates, 100 µl of (v/v) 0.05% glutaraldehyde in PBS (100 µl) was added and the plates were left for 15 min at 23°C. After this period the plates were washed three times with PBS and incubated (30 min, 23°C) with 0.1M glycine containing 0.1% (w/v) BSA. The wash step was repeated and culture medium containing 10% (v/v) FCS (100 µl) was added to each well and incubated (1h, 37°C). The plates were then either stored at

-20°C or used immediately.

1.2 Solid Phase Radioimmunoassay

The technique involved the use of iodinated Protein A to detect the binding of anti-(AChR) antisera to fixed PBL coated plates.

1.2.1 Iodination of Staphylococcus aureus Protein A

Protein A was labelled with [^{125}I] by the chloramine T method (Hunter, 1967) as modified by Urbaniak et al., (1973). Preparation of the Protein A prior to iodination was carried out as described by Hudson & Hay (1976). Protein A (0.5% (w/v) in 200 µl distilled water) was added to 0.12M carbonate-bicarbonate buffer, pH 9.0 (800 µl) and a solution of dioxane containing 10mg/ml hydroxyphenol succinimide (10 µl). The mixture was incubated (25 min, 23°C, with occasional mixing), and then dialysed against PBS (2L, 16h, 4°C). Protein A, so treated, was iodinated by the same method used for the iodination of α-BGT (Methods, Section A1.1).

1.2.2 Determination of the biological activity of [^{125}I]-Protein A

The biological activity of [^{125}I]-Protein A can be assessed by its ability to bind IgG. However, Protein A does not form a precipitable immune complex and it is necessary to immobilise the IgG onto a solid support such as nitrocellulose paper or Sepharose beads. These methods were used to assess the biological activity of the [^{125}I]-Protein A preparations.

Human IgG (2mg/ml) was spotted (1 µl volume) onto nitrocellulose paper circles, which had been equilibrated in the dilution

buffer 50mM Tris-HCl buffer, pH 7.4 containing 200mM NaCl to give the range of concentrations 10ng/μl, 100ng/μl, 1μg/μl and 2μg/μl. BSA at the same concentrations was used also to spot the nitrocellulose paper in order to determine non-specific binding. All dilutions were made in the dilution buffer. The paper circles dotted with immunoglobulin were dried at 37°C for 10 min and then incubated with a range of dilutions of [^{125}I]-Protein A (1/10, 1/50, 1/100 in dilution buffer, 100μl). Following incubation (2h, 37°C), with occasional mixing, the paper circles were washed extensively with the dilution buffer, dried and then counted for radioactivity.

The second method used rabbit-IgG conjugated to Sepharose beads. Increasing amounts of rabbit IgG conjugated to ACA-22 beads (200μl; 1μl of beads corresponding to 575ng of rabbit IgG) were incubated for 90 min at 37°C with [^{125}I]-Protein A (0.1nmol, 100μl). The beads were then washed with PBS (2 x 3ml) by centrifugation (high speed, 4°C, 5 min; Beckmann microfuge) and the radioactivity bound to the beads was determined. Non-specific binding of [^{125}I]-Protein A was determined by using non-IgG conjugated ACA-22 beads.

1.2.3 Solid Phase Radioimmunoassay protocol

The fixed PBL-coated plates (Section D1.1) were washed twice with PBS before the addition and incubation of diluted serum or antiserum (100μl/well; 2h, 37°C). The antisera used were rabbit anti-(Torpedo AChR) (designated T), two rabbit anti-(fetal calf AChR) antisera (designated F1 & F2), rabbit

anti-human IgG, rabbit anti-BSA and NRS. All dilutions of the sera were made in wash buffer (PBS containing 2% w/v) BSA and 0.002% (w/v) sodium azide). Tests were made in triplicate for each dilution and some wells were incubated with wash buffer only to assess background values. After incubation, the plates were washed three times with wash buffer and [^{125}I]-Protein A (100 µl/well) was added and left for 90 min at 4°C. The plates were then washed six times with wash buffer and the individual wells were cut up and counted for radioactivity.

1.3 ELISA using fixed PBL coated plates

The method is exactly the same as that used for the Solid Phase RIA except that the binding of antisera to the fixed PBL was detected by the means of an enzyme-labelled second antibody (alkaline phosphatase conjugated goat anti-rabbit IgG; 100 µl/well, diluted 1/1000 in PBS containing 2% (w/v) BSA). After incubation (90 min, 23°C) the plates were extensively washed with the above buffer and substrate solution (100 µl; 1mg/ml p-nitrophenylphosphate in 0.1M glycine buffer pH 10.4 containing additionally 1mM $MgCl_2$ and 1mM $ZnCl_2$) was added. The absorbance at 405nm was read after 30 min using a Titretek ELISA reader.

2. <u>Immunoprecipitation of iodinated PBL membranes by anti-(AChR) antisera and Mabs</u>

Human PBL were iodinated using a lactoperoxidase-catalysed reaction and the iodinated membranes solubilised and used in immunoprecipitation assays with anti-(AChR) antisera and Mabs.

2.1 Lactoperoxidase-catalysed iodination of lymphocyte plasma membranes

Normal PBL (viability >95%) were isolated as described in Methods, Section D1 and were washed 3 times by centrifugation (200g, 10 min) using PBS to remove extracellular protein. PBL (5 - 10 x 10^7 cells) were then finally resuspended in PBS (150 µl) and placed in a water bath at 30°C. Lactoperoxidase solution (0.25mg/ml/PBS, 10 µl) was then added, followed by the addition of PBS (20 µl) and Na [^{125}I] (10 µl, 1mCi). The lactoperoxidase-catalysed iodination reaction was started by the addition of H202 (0.03% (v/v)/ PBS, 10 µl), followed immediately by vigourous mixing. The reaction mixture was then incubated (4 min, 30°C) before the addition of H202 (0.03% (v/v) in PBS, 10 µl). The incubation step and the addition of H202 was repeated once more, before the reaction was terminated by adding PBS (5ml, 4°C). The reaction mixture was centrifuged (200g, 10 min, 4°C) and the resulting supernatant was collected. The lymphocyte pellet was washed with PBS (5ml, 4°C), centrifuged as before, and again the supernatant was collected. Samples (5 µl) of the two resulting supernatants were kept for counting in order to estimate free [^{125}I]. The lymphocyte pellet was resuspended in PBS (1ml, 4°C) and a sample (5 µl) was taken for counting to assess the radioactivity associated with the lymphocyte membrane.

2.2 Solubilisation of [^{125}I]-lymphocytes

The [^{125}I]-lymphocytes were solubilised by using the detergent Nonidet-P40 (NP-40). All manipulations were carried out at 4°C.

The iodinated lymphocyte suspension was again centrifuged (200g, 10 min, 4°C) and to the pellet was added 0.05% (w/v) NP-40 in 10mM Tris/NaCl buffer pH 7.2 containing the protease inhibitors, 10mM EDTA, 20mM iodoacetamide and 1mM PMSF. The solubilisation step was carried out for a total of 30 min with vigorous mixing every 10 min. The mixture was then centrifuged (high speed, 5 min, 4°C, Beckman bench centrifuge) and the supernatant was removed and tested for trichloroacetic acid (TCA) insoluble radioactivity.

2.3 Trichoroacetic acid precipitation

Prior to TCA precipitation, the extract was diluted (1/100) in PBS containing 5% BSA (w/v). Samples (100 µl) of the diluted extract were added to 10% (w/v) TCA solution (100 µl) and left to incubate for 90 min at room temperature. The solutions were then filtered through GFC filters and the filters were washed (2 x 5ml) with 5% (w/v) TCA. The filters were then dried and counted for radioactivity.

The percentage incorporation of radioactivity into protein was then calculated as follows :-

$$\% \text{ incorporation} = \frac{\text{cpm filter after TCA precipitation}}{\text{cpm of 100 µl (1/100 labelled membranes)}} \times 100$$

2.4 Immunoprecipitation assay

This assay used Eppendorf tubes which had been incubated (24h, 37°C) with 1% (v/v) normal serum in PBS from the same species as the origin of the second immunoprecipitating antibody. The tubes were then washed 3 times with PBS.

The iodinated lymphocyte membrane extract was diluted (1/5–1/10) in NP-40 washing buffer comprising 0.05% (w/v) NP-40, 0.05 M Tris-HCl buffer, pH 7.2 containing 0.15M NaCl, 5mM EDTA, 1% (w/v) casein, 10mM KI and 0.02% (w/v) sodium azide and incubated (overnight, 4°C) in 100 µl volumes (representing 5×10^5 iodinated PBL) in a final volume (500 µl) with control or test antibody.

Cross reactivity studies were carried out by using Mab culture supernatants (200 µl) from B11, C7, C11, E8 and E11. Here, NMS (10 µl) was added as a carrier protein. Ascites fluid (10µl) from Mabs B11, C7, C11, E8 and E11 were also used, with anti-neurofilament and anti-BSA ascites fluid and NMS as controls. The cross reactivity of rabbit anti-(AChR) antisera (5 µl) F1, F2 & T was also studied using anti-BSA and anti-(human Fabµ) as controls.

Immunoprecipitates were formed after the addition of appropriate second antibody (goat anti-mouse IgG, 70 µl; goat anti-rabbit IgG, 135 µl) and an incubation period of 2h at 37°C. The immunoprecipitates were collected by centrifugation, washed three times with ice-cold washing buffer (3 x 1ml, high speed, 2 min, 4°C, Beckman microfuge) and then counted for radioactivity. All determinations were made in triplicate.

RESULTS

1. Solid Phase Radioimmunoassay

1.1 Iodination and biological activity of Protein A

1.2 Cross-reactivity studies using polyclonal anti-(AChR) antisera

2. Cross-reactivity of anti-AChR antisera by ELISA

3. Immunoprecipitation studies

3.1 Iodination of lymphocyte membranes and extract preparation

3.2 Immunoprecipitation of [^{125}I]-PBL membrane extract by anti-(AChR) antibodies

This section examines the cross-reactivity of both polyclonal anti-(AChR) antisera, T1, F1 and F2 (see Sections B and C) and the Mabs C7, E11, B11, C11 and E8 with human PBL.

The studies using polyclonal antisera were carried out before the availability of the Mab probes and cross-reactivity was assessed by Solid Phase RIA using iodinated Protein A and ELISA using enzyme conjugated second antibody as detecting signals.

1. Solid Phase Radioimmunoassay

This method used fixed human PBL (Methods, Section E1.1) as targets for rabbit-anti-(AChR) antisera and control rabbit sera.

[^{125}I]-protein A was used to detect any binding of the rabbit serum samples to the PBL.

1.1 Iodination and biological activity of Protein A

The elution profile of [^{125}I]-Protein A from the G25 Sephadex column (Methods, Section E1.2.1) was similar to that shown for the iodination of α-BGT as shown in Figure 8 (Results Section A1.1). Three iodinations of Protein A were carried out and each gave 93-94% incorporation of [^{125}I] (see Table 45). However, retention of biological activity after treatment with the oxidising agent chloramine T, used during the iodination process was a problem. The first preparation of [^{125}I]-Protein A proved to have a low biological activity (3%) (see Table 45). The iodination protocol was, accordingly modified for the two

Table 45 Iodination of Protein A

Iodination No.	Ratio Protein A (nmoles) ^{125}I -Na(mCi)	Reaction time with Chloramine T	Specificity Activity Ci/mmol	% incorporation of ^{125}I	Biological Activity
1	0.475 nmoles/mCi	60 secs	1980	94%	[a] 3%
2	3.80 nmoles/mCi	30 secs	246	93%	[a] >16%
3	4.75 nmoles/mCi	30 secs	198	94%	[b] 69%

a The biological activity of the preparations was assessed using immobilised human IgG on nitrocellulose paper (Methods, Section E 1.2.2)

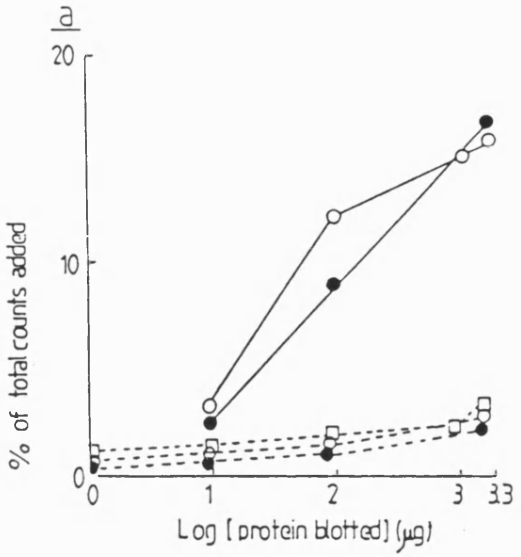
b The biological activity was determined using Rabbit IgG conjugated to ACA-22 beads (Methods, Section E 1.2.2)

following iodinations. These modifications involved reduction of the reaction time (60S - 30S) between the protein and [^{125}I]-Na in the presence of chloramine T, and also increasing the Protein A to [^{125}I]-Na ratio, from 0.475 nmoles/mCi to a maximum 4.75 nmoles/mCi. This resulted in a reduction of the specific activity of the preparations from 1980 Ci/mmol to 198 Ci/mmol but increased the biological activity of the preparations (see next section).

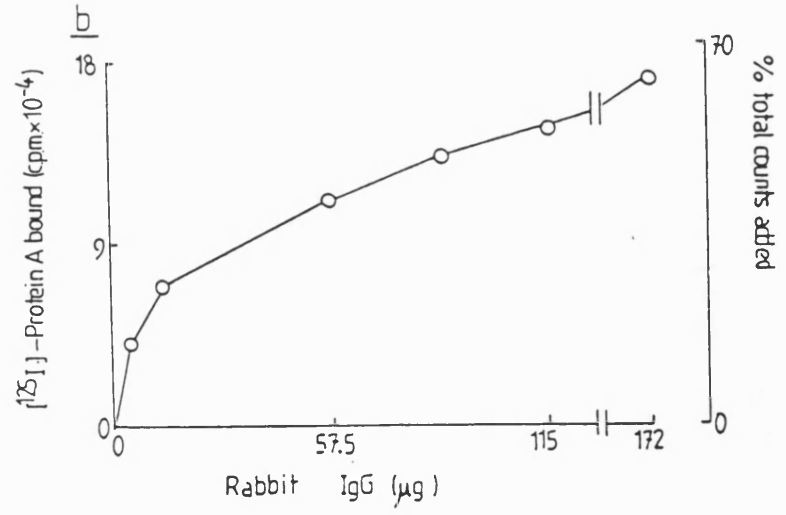
The biological activity of [^{125}I]-protein A preparations 1 and 2 was assessed by the binding of the diluted preparations to nitrocellulose circles dotted with known amounts of human IgG (Methods, Section E1.2.2). Using this method, [^{125}I]-Protein A from iodination 1 showed little binding to the human IgG (maximum 3% of the total counts added using 2μg human IgG). Accordingly, this preparation was not used in the radioimmunoassay. However, preparation 2 had a much greater biological activity of greater than 16%, using 2μg human IgG (see Table 45 and Figure 51 a). It was, however, difficult to accurately determine the biological activity of the iodinated preparations using this method; the binding of preparation 2 was not saturating (Figure 51 a) and it was difficult to dot more immunoglobulin onto the circles. Because of this, an alternative assay method was used to determine the biological activity of preparation 3.

This method used rabbit IgG conjugated to ACA-22 beads and was advantageous because the amount of IgG used was simply a factor

Figure 51 The Biological Activity of [^{125}I]-Protein A



a. The biological activity of two [^{125}I]-protein A preparations, (- - -, iodination 1; ——— iodination 2) were assessed by incubating nitrocellulose circles dotted with varying amounts of human IgG with diluted [^{125}I]-Protein A (□, 1/10; ●, 1/50; ○, 1/100 dilutions) as described in Methods, Section E1.2.2.



b. The biological activity of [^{125}I]-protein A (iodination 3) was assessed by incubating varying amounts of beads coated with rabbit IgG with diluted [^{125}I]-protein A (1/50, equivalent to approximately 26,000 cpm) as described in Methods, Section E 1.2.2.

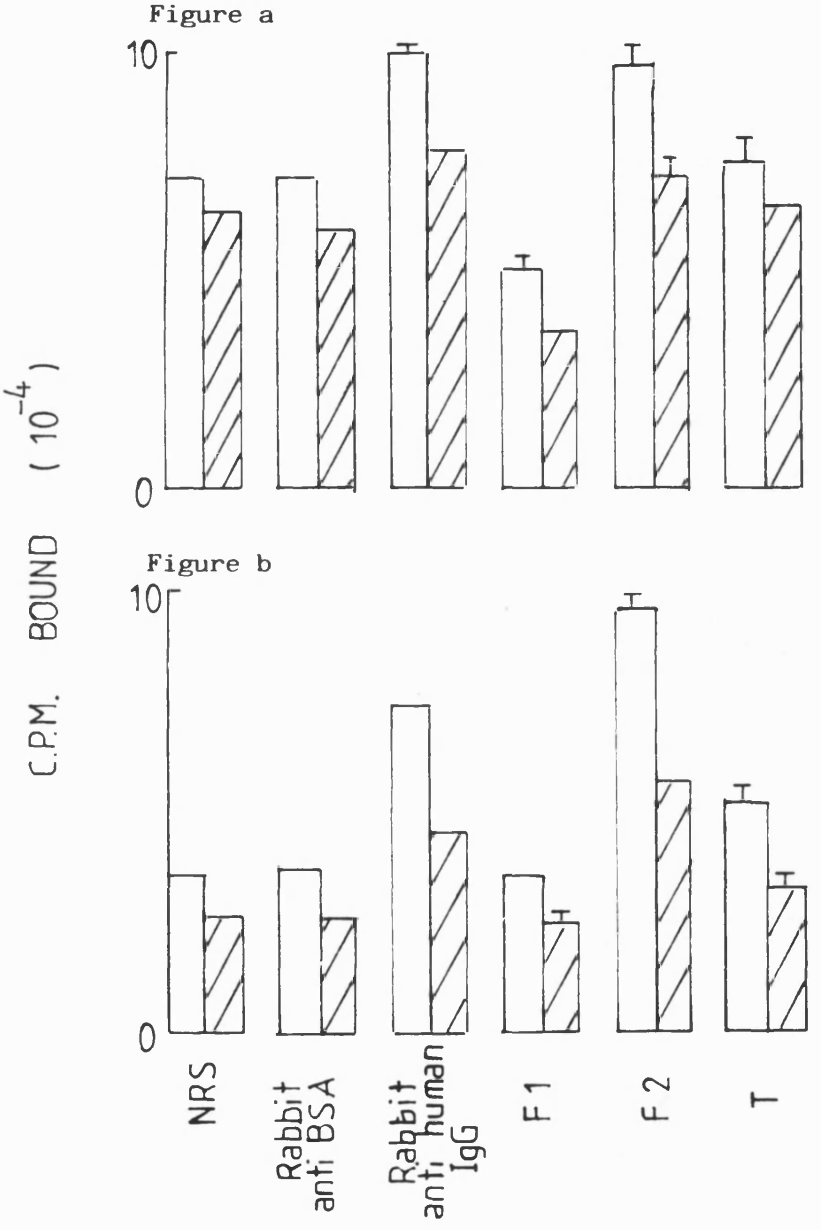
of how many beads were used in the incubation step with [^{125}I]-Protein A. This method gave a biological activity of 69% for [^{125}I]-Protein A preparation 2 (Figure 51 b).

1.2 Cross-reactivity studies using polyclonal anti-(AChR) antisera

[^{125}I]-Protein A preparations 2 and 3 were used to detect the binding of anti-(AChR) antisera (T, F1 and F2) to fixed monolayers of human PBL (Methods, Section E1.2.3). Rabbit anti-(human IgG) antiserum provided a positive control, while NRS and rabbit anti-(BSA) antiserum were negative controls. The binding of [^{125}I]-Protein A to human PBL in the absence of sera was determined to control for binding to surface immunoglobulin on the PBL. Using PBL from four different individuals, this binding represented 0.52 $\pm$ 0.1% (mean $\pm$ S.E.) of the total radioactivity added.

The first cross-reactivity studies used serum samples at dilutions of 1/2 and 1/4 and the results are shown in Figures 52 a and b. The assay was reproducible; the errors between triplicates were small, being 0.1 - 2.5% of the specific cpm bound. The binding of the antisera to human PBL was assessed by analysis of variance. The binding of antiserum F1 to human PBL was not significantly different from that of NRS and rabbit anti-(BSA) sera, whereas the binding of antisera F2 and rabbit anti-(human IgG) gave significantly increased binding ($p < 0.01$). The binding of the antiserum T was significantly different from the negative controls ($p < 0.01$) in Figure 52 b only.

Figure 52 Cross-reactivity of Rabbit anti-(AChR) antisera with human PBL by SOlid Phase Radioimmunoassay



The cross-reactivity of the sera was determined at two dilutions (□ , ½; ▨ , ¼) against fixed human PBL from two different individuals Binding was detected by [^{125}I]-Protein A (preparation 2; Figure a 173264 cpm; Figure b 140685 cpm was added). Binding of [^{125}I]-Protein A in the absence of sera was 1269 and 1490 cpm for figures a and b respectively).

In titration curves (Figures 53a and 53b) the binding of antisera F2 and rabbit anti-(human IgG) was significantly different ($p < 0.01$) from that of NRS and rabbit anti-(BSA) antisera at all the dilutions used.

The IgG concentrations of the antisera T1, F1 and F2 were 14.2, 13.8 and 12.8 mg/ml respectively. The control sera NRS, rabbit anti-BSA and rabbit anti-(human IgG) contained 11.8, 13.1 and 12.4 mg/ml, respectively (determinations were carried out by a colleague).

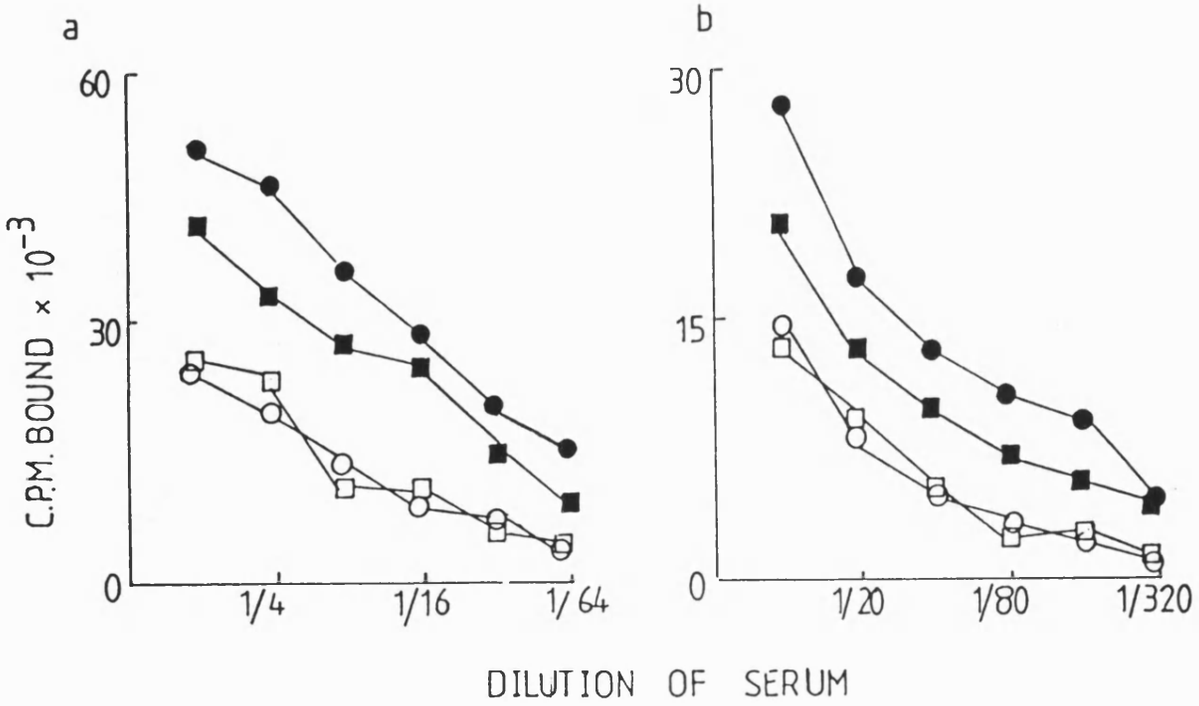
2. Cross-reactivity of anti-AChR antisera by ELISA

The cross-reactivity of the anti-(AChR) antisera, F1. F2 and T with fixed human PBL from three different individuals was detected by an ELISA system as described in Methods, Section E1.3. Errors for each determination made in triplicate represented 2.5% - 10% of the optical density measured. For each PBL population studied antisera F2 gave higher binding than the other antisera and control sera, NRS and anti-(BSA) antisera (greater than three standard deviations of control binding; Figure 54).

3. Immunoprecipitation studies

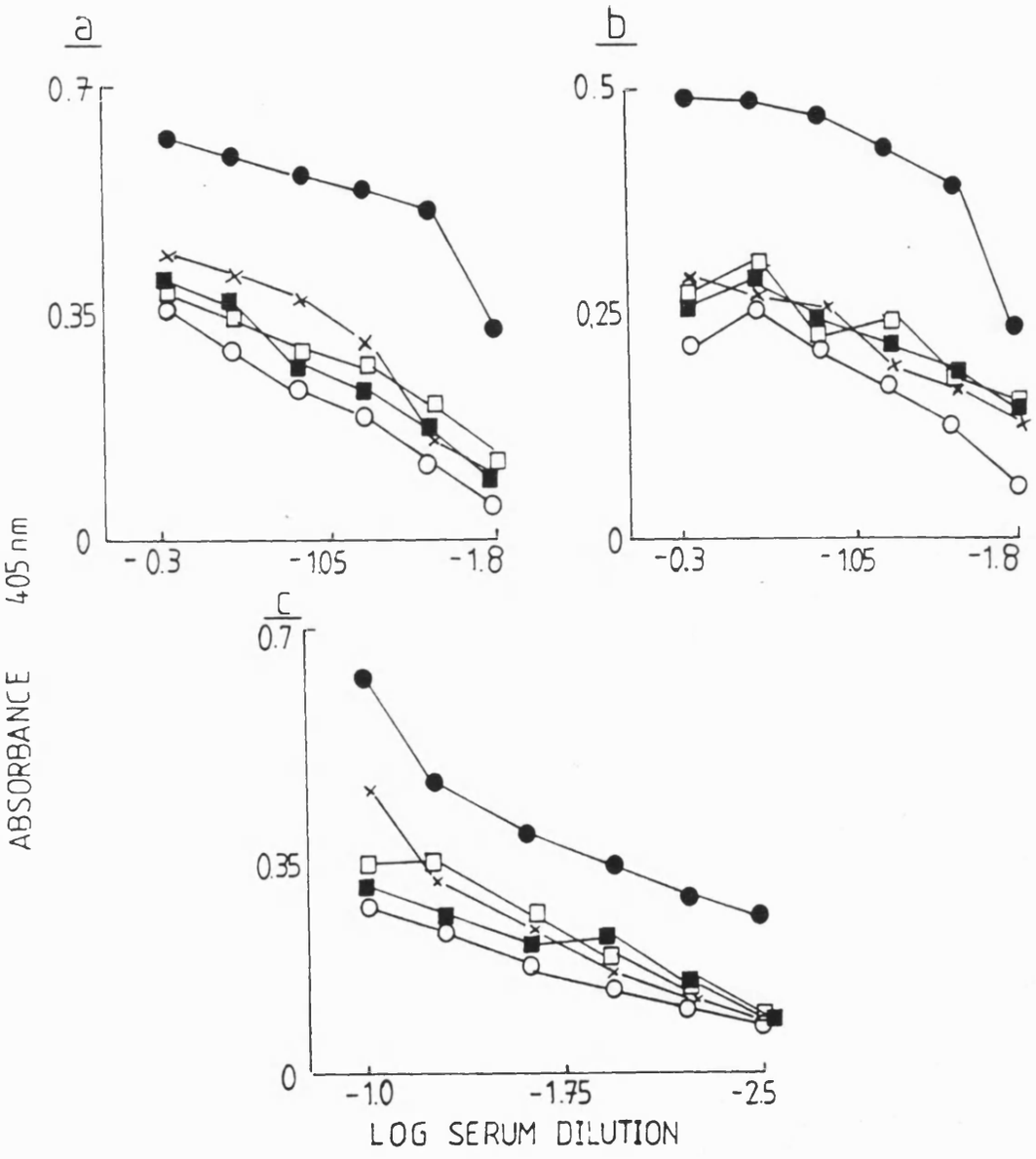
The extracellular surface of PBL was labelled with [^{125}I] by a lactoperoxidase catalysed reaction (Methods, Section E2.1). The iodinated PBL were then solubilised by using detergent (Methods, Section E2.2) and then the ability of polyclonal and monoclonal

Figure 53 Cross-reactivity of Rabbit anti-(AChR) antisera F2 with human PBL by Solid Phase Radioimmunoassay



Varying dilutions of NRS, (-○-); rabbit anti-(BSA), (-□-); rabbit anti-(human IgG), (-●-) and antisera F2 (-■-) were incubated with PBL from 2 different individuals (a and b). Binding was detected by [^{125}I]-protein A (preparation 3; figure a 133280 cpm; figure b 123180 cpm). Binding of [^{125}I]protein A in the absence of sera was 720 and 1112 cpm for figures a and b respectively.

Figure 54 Cross-reactivity of anti-AChR antiserum with fixed human PBL by ELISA



ELISA wells coated with fixed human PBL from three different individuals; Figures a, b and c; were incubated with dilutions of NRS (-o-); rabbit anti-BSA, (-■-); antiserum F1 (-×-); antiserum F2 (-●-); antiserum T (-□-). The plates were then incubated with alkaline phosphatase conjugated second antibody and developed as described in Methods, Section E 1.3.

Each point is the mean of a determination made in triplicate.

anti-(AChR) antibodies to immunoprecipitate the iodinated membranes was assessed (Methods, Section E2.4).

3.1 Iodination of lymphocyte membranes and extract preparation

Human PBL were iodinated by using a lactoperoxidase-catalysed reaction (Methods, Section E2.1). The lactoperoxidase enzyme cannot cross the plasma membrane of viable cells and only surface components are iodinated. Hence, only PBL preparations with high viability were used. The incorporation of [^{125}I] varied with each iodination (see Table 46), with 85-99% of these counts present in the extract after solubilisation of the cell membranes. The incorporation of [^{125}I] into protein was determined by TCA precipitation (Methods, Section E2.3) and varied from 2-9% of the total radioacitivty of the extract.

3.2 Immunoprecipitation of [^{125}I]-PBL membrane extract by anti-(AChR) antibodies

The ability of polyclonal and monoclonal anti-(AChR) antibodies to immunoprecipitate [^{125}I]-PBL membrane extracts was assessed (Methods, Section E2.4).

In experiments using polyclonal antisera only antisera F2 and rabbit anti-(human Fabμ) antisera gave binding significantly different from that of the controls (Table 47).

The initial experiments using Mabs used culture supernatants as the source of antibody. However, no differences between test and control supernatants were observed and it was decided to use ascites fluid, as this provided a much more concentrated form of antibody. In two experiments, using [^{125}I]-PBL from two

Iodination Number	No. of lymphocytes used for iodination	[a]% incorporation of ^{125}I	[b]% cpm extracted	Proportion of sample radioactivity bound to protein
1	9.8×10^7	8%	95%	9%
2	6.5×10^7	5%	85%	2%
3	1×10^8	25%	99%	8%

Table 46 Summary from the Lactoperoxidase catalysed iodination of lymphocyte membranes

The values were determined as follows:-

(a) cpm associated with cells as a % of the total cpm added
(b) cpm extracted as % of cpm associated with the cells
(c) TCA insoluble cpm as % of cpm of extract

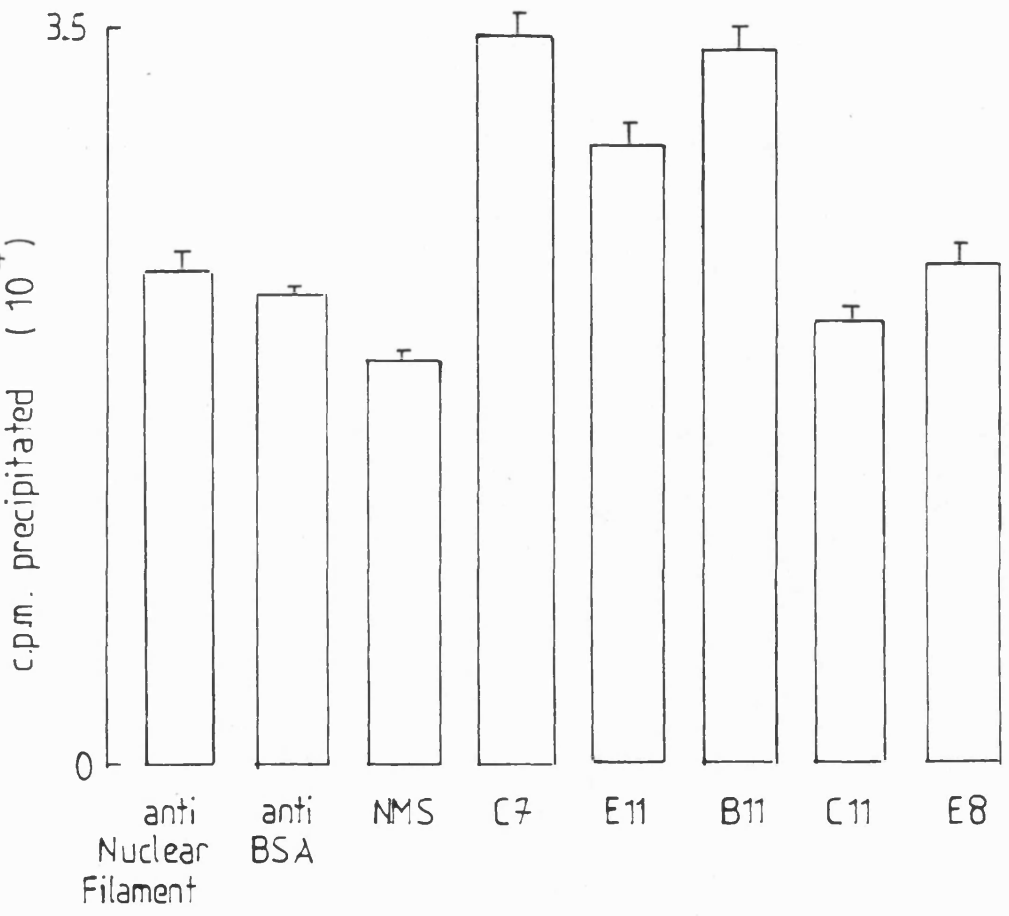
different individuals (preparations 1 and 3, Table 46), the Mabs C7, B11 and E11 gave binding significantly different ($p<0.01$) from that of the negative controls NMS, mouse anti-neuro filament and mouse anti-BSA (Figure 55).

Table 47 Immunoprecipitation of extract of [^{125}I] human PBL membranes by anti-(AChR) antisera

Serum Sample	Binding (c.p.m.) Mean ± S.E.
Rabbit normal serum	5034 ± 66
Rabbit anti-BSA	4909 ± 101
Rabbit anti human Fabμ	*6107 ± 203
Rabbit antisera F1	5374 ± 32
Rabbit antisera F2	*6572 ± 23
Rabbit antisera T	5428 ± 24

Rabbit serum samples were incubated with [^{125}I]-PBL membranes (preparation 1. 806444 cpm total) followed by the addition of goat anti-(rabbit IgG) as described Methods, Section E2.4. Background counts were 5645 cpm. Results are the mean ± S.E. of triplicate determinations. *Values significantly different from the control values (NRS and rabbit anti-(BSA) at $P < 0.01$).

Figure 55 Immunoprecipitation of $[^{125}I]$-PBL using anti-(AChR) Mabs



The results shown are the mean from two immunoprecipitation experiments using $[^{125}I]$-PBL preparations 1 and 3. Normal mouse serum (NMS), and the ascitic fluid anti-BSA and anti-nuclear filament were used as negative controls. Values significantly different from the control values were obtained with anti-(AChR) Mab ascitic fluid C7, B11 and E11.

DISCUSSION

Several groups have used polyclonal and monoclonal anti-(AChR) antibodies to investigate the presence of AChR epitopes on immune cells. However, the majority of these studies have used human (Horvat et al., 1983; Pizzighella et al., 1983; Kirchner et al., 1987) and mouse thymocytes (Fuchs et al., 1980; Horvat et al., 1983; Riviera et al., 1987) as target cells. The present study was primarily concerned with the presence of nAChR on human PBL which has been implicated by a number of studies (Table 48, page 292a).

Solid Phase Radioimmunoassay and ELISA techniques necessitated the use of immobilised PBL. Con A which binds to the surface glycoproteins of lymphocytes provided a reliable means of immobilising the cells onto mictotitre plates. Poly-l-lysine has also been successfully employed in Cell ELISA and this acts to increase the electrostatic attraction between the cells and the microtitre wells (Drover and Marshall, 1986). Fixation of the human PBL by a dilute solution of glutaraldehyde ensured no cell loss during the subsequent extensive washing and incubation procedures. However, because glutaraldehyde fixation has been shown to damage or destroy cell surface antigenic determinants, (Drover and Marshall, 1986), a dilute solution of glutaraldehyde and a short-fixation period were used, as recommended by Drover & Marshall, 1986 and references therein.

Iodinated Protein A provided a sensitive means of detecting cross-reactivity as Protein A readily reacts with all subclasses

Table 48 Studies implicating the presence of nAChRs on human PBL

Functional Studies	
Effect of nicotinic cholinergic ligands on PBL proliferation	Neher et al., 1974 Strom et al., 1974 Richman and Arnason, 1979 Richman et al., 1981 Menard and Rola-Pleszcznski, 1983
Reversal by d-tubocurarine of the inhibitory effect of carbachol on E-rosette formation	Mizuno et al., 1982a, b.
Abrogating effect of α-BGT on enhanced C2 production of monocytes by ACh	Whaley et al., 1981
Immunological cross-reactivity with anti-AChR antibodies	Shimizu, 1980 Arimori et al., 1981 Mischak and Dau, 1981
α-BGT binding to PBL	Morrell, 1979, 1981.

of rabbit IgG (Richman et al., 1982). The conditions of the iodination reaction appeared to be crucial. Loss of activity in the first preparation may have resulted from chemical damage caused by the harsh oxidising conditions promoted by chloramine T.

Other workers have shown that limiting amounts of chloramine T relative to the protein concentration and a short reaction time are critical for the conservation of biological activity (Langone, 1980) and this was also observed in this study (Results, Table 45). It appears that all four tyrosine residues of Protein A are necessary for maximum functional activity and that loss of Fc binding can result from the incorporation of more than one iodine atom per molecule (Langone, 1980). These factors could account for the low biological activity of preparation 1.

The use of beads coated with rabbit IgG proved to be a more precise indicator of the biological activity of the iodinated Protein A preparations than the immunodot method (Results, Section E1.1). The use of the former method was also more relevant to their subsequent use in detecting all human IgG subclasses (Richman et al., 1982). Hence, the use of this assay probably led to an underestimation of the biological activity of the first two iodinated Protein A preparations. Nevertheless, it was evident that the use of a modified iodination procedure for preparations 2 and 3 resulted in a concommitant increase in biological activity.

Both the Protein A and ELISA assays were reproducible and background noise, due to the binding of iodinated Protein A to Fc receptors on human PBL or cross-reactivity of conjugated second antibody with human PBL was negligible. Additionally there was no apparent problem with interference of the cell-coated plates with the measurement of optical density.

The immunoprecipitation studies provided another means of detecting cross-reacting epitopes on human PBL and was used as an alternative assay as the anti-(AChR) Mabs were of IgG1 subclass; a mouse sub-class not detected by Protein A. A lactoperoxidase catalysed iodination of human PBL was chosen because it was the mildest iodination procedure available. It is a highly controlled procedure, dependent on the amount of peroxidase added and it labels only the external surfaces of lymphocytes provided that highly viable cells are used.

Only a small percentage of the total added [^{125}I] was associated with protein components (Results, Table 46). It is likely that the majority of the counts represent free [^{125}I] with small amounts associated with glycolipid (see Johnstone and Thorpe., 1982). These components were not thought to interfere with the use of solubilised iodinated PBL membrane in immunoprecipitation studies, but they could have been removed by gel filtration.

The choice of control sera in such cross-reactivity studies is important as the immunoglobulin concentration or spectrum of sera from animals which have been hyperimmunised against antigen is not the same as that found in normal serum. There is usually

a significant increase in levels of IgG in immune sera. Hence, antisera raised against a non-AChR related antigen (anti-BSA) was used in the assays as an additional control and indeed slightly higher binding of anti-BSA antisera compared to NRS was observed in both solid phase RIA and ELISA assays (Results, Table 47 and Figure 52-54). For the same reasons, ascites fluid raised against antigens other than AChR were used in addition to NMS as negative controls in immunoprecipitation experiments.

In Protein A, ELISA and immunoprecipitation assays antisera F2 raised against fetal calf AChR consistently showed cross-reactivity with human PBL and, interestingly, this antiserum showed the highest cross-reactivity with human muscle AChR compared with the other anti-AChR antisera tested (Results, Section C1). Additionally, three Mabs raised against Torpedo AChR (Mabs C7, E11 and B11) showed significantly increased reactivity compared to the other Mabs and controls in immunoprecipitation experiments. All these antibodies showed cross-reactivity with human muscle AChR (Results, Section C1 and 2) although to different degrees. Direct and indirect evidence respectively indicated that Mab B11 and Mab C7 react with the α-subunit of Torpedo AChR (Discussion, Section C) and Kirchner et al., (1987) have previously shown cross-reactivity of anti-α-subunit Mabs with thymic AChR. However, in this present study two other α-subunit reactive Mabs E8 and C11 failed to show reactivity with human PBL. Two of the cross-reacting Mabs, C7 and E11, also appear to bind near the cholinergic binding site of AChR and Pizzighella et al., (1982) have shown that a

similar cholinergic site directed Mab can modify the function of an AChR present on mouse thymocytes. It is not known whether the thymic AChR component is related to a similar anti-AChR antibody binding protein on PBL. With respect to this, a study by Horvat et al., (1983) failed to show cross- reactivity of anti-(Torpedo AChR) antisera with human PBL, but could nevertheless, demonstrate cross-reactivity in the thymus, using immunofluorescence studies. Hence indicating that the cross--reacting epitopes are not the same.

It is important to emphasise that these cross-reactivity studies do not confirm the presence of AChR epitopes on human PBL. The exact specificity of the binding to human PBL should be checked in order to rule out the possibility that antibodies of other non-AChR specificities could react with human PBL. Similarly, there could be components other than anti-AChR antibodies present in ascites fluid. Additionally, there appear to be differences in the adhesiveness of the different classes of immunoglobulin, with IgM being the most 'sticky' (Drover and Marshall, 1986).

Verification of true reactivity could be carried out by using purified antibodies $F(ab)_2$ fragments or by specific absorption of anti-(AChR) antibodies by AChR-Sepharose adsorbent and the use of absorbed and non-absorbed antisera in parallel experiments. These studies were not carried out because of time considerations. Nevertheless, the methods used in this present work could be used to screen large libraries of anti-AChR

antisera and Mabs for reactivity with human PBL. Cross-reacting antibodies detected by these initial screening methods could then be more rigorously studied. The antisera F2, and Mabs C7, E11 and B11 would be good candidates in this respect. The immunoprecipitation of iodinated PBL membranes by cross-reacting antibodies has an important advantage over the other screening assays, as cross-reacting components can be analysed electrophoretically, hence giving useful information on the molecular weight of the cross-reacting protein. FACS analysis, as used by Fuchs et al., (1980) in the study of 'AChR like' antigens on mouse thymocytes would also be useful in these studies, however such expensive equipment is not always available.

GENERAL DISCUSSION AND PERSPECTIVES

The evidence for the presence of nAChR on cells of the immune system has previously relied on information obtained from functional assays. However, definitive proof of the presence of this receptor on human PBL depends upon the correlation of functional activity with a structurally defined receptor protein. In this work two approaches were used to look for nAChRs on human PBL: radioligand binding assays using [^{125}I]-α-BGT and [^{3}H](-)- nicotine and cross-reactivity studies with anti-(AChR) antibodies.

This work was, however, unable to demonstrate the presence of nicotinic cholinergic binding sites on human PBL (Section D). This may be due to the receptors being present in extremely low numbers and/or the possibility that the receptors are present on a particular subset or subsets of lymphocytes which may represent only a small proportion of the total lymphocyte population. The use of larger numbers of lymphocytes in the assay procedure may have overcome this problem. Investigation of the presence of nAChRs on certain lymphocyte subsets could be studied by using fractionated lymphocyte populations in the radioligand binding assays. If successful this work could be extended to the separation of the cells bearing nAChRs by cell affinity chromatography on α-toxin or nicotine derivatives attached to a solid support such as Sepharose 6B. However, these studies require a large source of lymphocytes and this is not always possible.

The presence of PBL membrane proteins that cross-reacted with anti-AChR antibodies was indicated in the latter studies of this thesis (Section E) and as above the use of fractionation techniques and antibody affinity columns may be useful in the purification of the receptor protein. Using immunofluorescent studies it should also be possible to determine whether the anti-AChR cross-reacting epitopes and the cholinergic binding sites represent the same protein entity.

Finally, another approach would be to make use of cDNA probes specific for AChR subunits to probe the mRNA of human PBL in cross-hybridisation experiments. Such an approach would provide a relatively clear-cut answer to this controversial subject and would then provide a more solid foundation for the study of functional and structural properties of this receptor.

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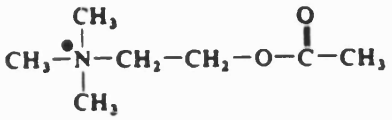
APPENDIX



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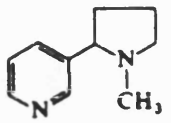

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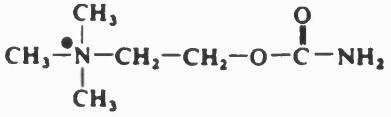

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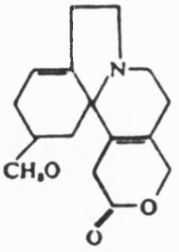

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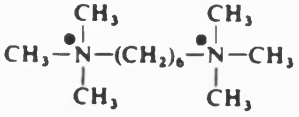

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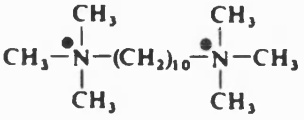

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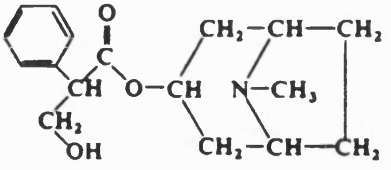

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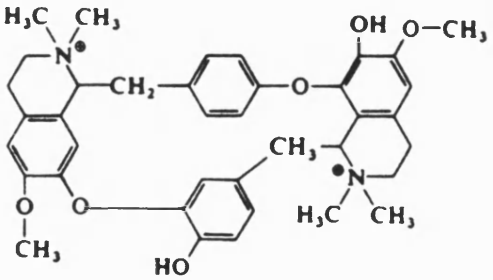

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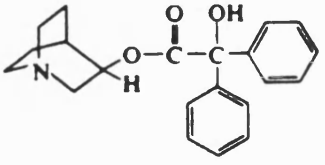

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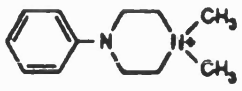
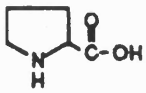


DMPP

Piperdine



Proline

Pyrrolidine